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(54) Title: COMBINATION FOR REDUCING ANTIMICROBIAL RESISTANCE USING A METHYLATION INHIBITOR IN COMBINATION WITH AN ANTIBIOTIC

(57) Abstract

Disclosed are novel methods, combinations of agents and kits for use in killing, or inhibiting the growth of microorganisms. Enhanced antimicrobial action is provided by using a methylation inhibitor, as exemplified by using an agent that inhibits methylation or maturation of bacterial RNA in combination with, e.g., a macrolide lincosamide streptogramin B (MLS) antibiotic. The methods and compositions described may be employed to reduce the resistance of susceptible microorganisms to antimicrobial agents and thus to treat animals or patients with infections.

DESCRIPTION

COMBINATION FOR REDUCING ANTIMICROBIAL RESISTANCE USING A METHYLATION INHIBITOR IN COMBINATION WITH AN ANTIBIOTIC

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to the fields of bacteria, antimicrobials and antibiotics. More particularly, it provides novel methods, kits, and combinations of antimicrobial agents and inhibitors, for use in reducing the resistance of bacteria and other microorganisms to antimicrobial agents. In particular, the invention provides for enhanced bacterial killing using a macrolide, lincosamide and streptogramin B (MLS) antibiotic in combination with an agent that inhibits methylation, as exemplified by inhibiting methylation or maturation of bacterial RNA.

Description of the Related Art

The first antibiotics were used clinically in the 1940s and 1950s, and their use has been increasing significantly since this period. Although an invaluable advance, antibiotic and antimicrobial therapy suffers from several problems, particularly when strains of various bacteria appear that are resistant to antibiotics. Interestingly, bacteria resistant to streptomycin were isolated about a year after this antibiotic was introduced (Waksman, 1945).

The development of antibiotic resistance is a serious and life-threatening event of worldwide importance. For example, strains of *Staphylococcus* are known that are immune to all antibiotics except one (Travis, 1994). Such bacteria often cause fatal hospital

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infections. Among other drug resistant organisms are:
pneumococci that cause pneumonia and meningitis;
Cryptosporidium and *E. coli* that cause diarrhea; and
enterococci that cause blood-stream, surgical wound and
5   urinary tract infections (Berkelman et. al., 1994).

      Davies (1986) described seven basic biochemical
mechanisms for naturally-occurring antibiotic resistance:
(1) alteration (inactivation) of the antibiotic; (2)
10   alteration of the target site; (3) blockage in the
transport of the antibiotic; (4) by-pass of the
antibiotic sensitive-step (replacement); (5) increase in
the level of the inhibited enzyme (titration of drug);
(6) sparing the antibiotic-sensitive step by endogenous
15   or exogenous product; and (7) production of a metabolite
that antagonizes action of inhibitor.

      Certain bacteria become resistant to antibiotics by
utilizing ribosomal mutations (Cunliffe, 1990), although
20   some reports have stated that this type of resistance is
of doubtful clinical significance (Spratt, 1994).
Ribosomal mutations result in bacterial resistance to
macrolide, lincosamide and streptogramin B (MLS)
antibiotics, as has been observed in the resistance of
25   various Staphylococcus, Streptococcus, Enterococcus,
Bacillus and Mycoplasma strains to important antibiotics
such as erythromycin (LeClercq & Courvalin, 1991).

      The induction of erythromycin resistance generally
30   leads to bacterial strains that express rRNA which does
not bind to this type of antibiotic. Mainly,
erythromycin resistance is due to the induction by
macrolides of a methylase protein, which catalyzes the
methylation of the binding site of erythromycin on the
35   rRNA, thus preventing antibiotic binding. Despite
intensive studies in this area, there remains few, if

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any, practical proposals as to how bacterial resistance
to MLS antibiotics may be overcome.

SUMMARY OF THE INVENTION

5

The present invention seeks to overcome these and
other drawbacks inherent in the prior art by providing
new combined compositions, medicaments, kits and uses
thereof in reducing resistance to antimicrobials and
10   antibiotics and for treating infections. The invention
rests in the surprising use of one or more inhibitors of
methylation in conjunction with an antimicrobial agent.
The combination is used to impair the ability of a
microorganism to methylate various substrates.

15

As the inventors contemplate that methylation,
either at the DNA, RNA, protein or small molecule level,
plays a role in all mechanisms of resistance of a
microorganism to an antimicrobial agent, inhibition of
20   methylation will be widely applicable to reducing
resistance in any given microorganism or bacterium, and
to treating a variety of diseases. For example, the
organisms listed in Table 1 and Table 2 may be targeted
using the antibiotics also listed in Table 1 and Table 2,
25   amongst others, in combination with a methylation
inhibitor. However, the invention is not limited to
antibiotics and to bacteria alone, and is widely
applicable to all microorganisms, including yeast and a
variety of other pathogenic and even parasitic organisms.
30

The invention therefore encompasses compositions,
medicaments, kits and uses thereof which include a
methylation inhibitor capable of inhibiting any process
listed in Table 1, as exemplified by the processes of
35   antibiotic alteration, target site alteration and
antibiotic transport. Also, as methylation inhibitors
act to alter gene expression and protein synthesis, the

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invention includes methylation inhibitors that prevent
the by-pass of antibiotic sensitive-steps, prevent an
increase in the amount of an inhibited enzyme, prevent
the production of an alternative endogenous or exogenous
5   product, and prevent the production of a metabolite that
antagonizes the action of inhibitor.

        One particular example of the applicability of the
invention is in providing combinations and medicaments
10   for use in reducing the resistance of bacteria to
macrolide, lincosamide, and streptogramin B (MLS)
antibiotics, or in enhancing the sensitivity of
susceptible strains to such antibiotics. In this case,
the inhibitor will primarily inhibit methylation of
15   bacterial rRNA, thus rendering the bacterium susceptible
to antibiotic rRNA binding and allowing bacterial protein
synthesis to be inhibited.

        The various embodiments of the invention include
20   combined compositions, medicaments and kits for use in
reducing the resistance of a microorganism to an
antimicrobial agent, as exemplified by reducing the
antibiotic resistance of an MLS-susceptible bacterium to
an MLS antibiotic. Also provided are compositions,
25   medicaments and therapeutic kits for killing
microorganisms, such as bacteria, and particularly,
MLS-susceptible bacteria; and their use for treating
infections and diseases caused by microorganisms,
including treating MLS-susceptible bacterial infections
30   and diseases. By way of example only, certain infections
that may be treated using the invention are listed in
Table 3.

        To reduce the resistance of a microorganism to an
35   antimicrobial agent, as exemplified by reducing the
resistance of a bacterium to an antibiotic, or to kill a
microorganism or bacterium, one would generally contact

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the microorganism or bacterium with an effective amount
of the antibiotic or antimicrobial agent in combination
with an amount of a second agent effective to inhibit
methylation in the microorganism or bacterium. In terms
5   of killing or reducing the resistance of an
MLS-susceptible bacterium, one would contact the
bacterium with an effective amount of an MLS antibiotic
in combination with an amount of a second agent effective
to inhibit RNA methylation, synthesis and/or maturation
10   in the bacterium. The terms "microorganism" and
"bacterium" are used for simplicity and it will be
understood that the invention is suitable for use against
a population of microorganisms, i.e., "bacteria".

15   The microorganism, e.g., bacterium, or population
thereof, may be contacted either *in vitro* or *in vivo*.
Contacting *in vivo* may be achieved by administering to an
animal (including a human patient) that has, or is
suspected to have a microbial or bacterial infection, a
20   therapeutically effective amount of pharmacologically
acceptable antimicrobial agent formulation in combination
with a therapeutic amount of a pharmacologically
acceptable formulation of a second agent effective to
inhibit methylation, e.g., effective to inhibit RNA
25   methylation. The invention may thus be employed to treat
both systemic and localized microbial and bacterial
infections by introducing the combination of agents into
the general circulation or by applying the combination,
e.g., topically to a specific site, such as a wound or
30   burn, or to the eye, ear or other site of infection.

      An "effective amount of an antimicrobial agent or
antibiotic" means an amount, or dose, within the range
normally given or prescribed. Such ranges are well
35   established in routine clinical practice and will thus be
known to those of skill in the art. Appropriate oral and
parenteral doses and treatment regimens are further

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detailed herein in Table 4 and Table 5. Supplementary
information relating specifically to MLS antibiotics is
also given in Example I and in Tables 6 and 7, which
particularly concern erythromycin, lincomycin,
clarithromycin and azithromycin. As this invention
provides for enhanced microbial and/or bacterial killing,
it will be appreciated that effective amounts of an
antimicrobial agent or antibiotic may be used that are
lower than the standard doses previously recommended when
the antimicrobial or antibiotic is combined with a
methylation inhibitor.

The "second agents" for use in the invention are
generally methylation inhibitors, and are also referred
to herein as "inhibitors" and "modifiers". The second
agent inhibitors should be used in amounts effective to
inhibit methylation in a microorganism or bacterium, as
exemplified by an amount effective to inhibit RNA
methylation, synthesis and/or maturation in an MLS-
susceptible bacterium. Suitable amounts effective to
inhibit methylation will be known, or readily
identifiable, to those of skill in the art. Effective
inhibitory amounts are the amounts that have previously
been shown in the scientific literature to inhibit
methylation generally or to inhibit a specific
methylation step. In addition to the present disclosure
and the references specifically incorporated herein,
there is considerable scientific literature concerning
methylation inhibitors that may be utilized in light of
the inventors' discovery that such compounds may be
effectively combined with antibiotics and other
antimicrobial agents.

Amounts effective to inhibit methylation may also be
measured, rather than identified from the published
literature. Most simply, this is achieved by determining
the amount effective to increase microbial or bacterial

killing when used in combination with an antimicrobial
agent, i.e., by determining an amount effective to reduce
antimicrobial resistance. The determinations of
effective inhibitory amounts and therapeutic doses will
be routine to those of skill in the art given the
teachings of the present disclosure, including the
detailed methodology and the effective amounts of various
agents disclosed, e.g., in Table 8 and throughout the
detailed examples.

Naturally, in confirming the optimal therapeutic
dose, first animal studies and then clinical trials would
be conducted, as is routinely practiced in the art.
Animal studies are common in the art and are further
described herein (Example II) and in publications such as
Lorian (1991, pp. 746-786, incorporated herein by
reference) and Cleeland & Squires (incorporated herein by
reference, from within the Lorian text).

The ID_{50}/IC_{50} ratio required for safe use of the
proposed inhibitor-antimicrobial agent combinations will
be assessed by determining the ID_{50} (median lethal toxic
dosage) and the IC_{50} (median effective therapeutic
dosage) in experimental animals. The optimal dose for
human subjects is then defined by fine-tuning the range
in clinical trials. In the case of ID_{50}, the inhibitor
is usually administered to mice or rats (orally or
intraperitoneal) at several doses (usually 4-5) in the
lethal rage. The dose in mg/kg is plotted against %
mortality and the dose at 50% represents the ID_{50}
(Klaassen, 1990). The IC_{50} is determined in a similar
fashion as described by Cleeland & Squires (1991).

In a clinical trial, the therapeutic dose would be
determined by maximizing the benefit to the patient,
whilst minimizing any side-effects or associated
toxicities. Throughout the detailed examples, various

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therapeutic ranges are listed. Unless otherwise stated,
these ranges refer to the amount of an agent to be
administered orally.

5   In optimizing a therapeutic dose within the ranges
disclosed herein, one would not use the upper limit of
the range as the starting point in a clinical trial due
to patient heterogeneity. Starting with a lower or mid-
range dose level, and then increasing the dose will limit
10   the possibility of eliciting a toxic or untoward reaction
in any given patient or subset of patients. The presence
of some side-effects or certain toxic reactions *per se*
would not, of course, limit the utility of the invention,
as it is well known that most beneficial drugs also
15   produce a limited amount of undesirable effects in
certain patients. Also, a variety of means are available
to the skilled practitioner to counteract certain side-
effects, such as using vitamin B_{12} in association with
N_2O treatment (Ostreicher, 1994).
20

It is important to note that the invention does not
require the identification of novel methylation
inhibitors, but rather concerns the new and surprisingly
effective use of various compounds, already known to have
25   certain functional properties, in combination with
antimicrobial agents or antibiotics. Neither does the
invention require the identification of a precise
molecular target, or of all targets, that a given
methylation inhibitor may act upon.
30

It is not necessary therefore to determine
methylation levels in a microorganism or bacterium to
practice the invention, or to identify all the enzymes or
other target molecules that are altered by the action of
35   the inhibitor. All that is required is to use an
antimicrobial agent in combination with an agent known to

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be, or suspected to be, capable of inhibiting methylation
and to determine whether increased killing results.

5   Zak & Sande (1981) reported on the correlation
between the *in vitro* and *in vivo* activity of a 1000
compounds that were randomly screened for antimicrobial
activity. The important finding in this study is that
negative *in vitro* data is particularly accurate, with the
negative *in vitro* results showing more than a 99%
10   correlation with negative *in vivo* activity. This is
meaningful in the context of the present invention as one
or more *in vitro* assays will be conducted prior to using
any given combination in a clinical setting. Any
negative result obtained in such an assay will thus be of
15   value, allowing efforts to be more usefully directed.

Although the invention was developed, in part, from
a consideration of various biochemical interactions and
pathways, an understanding of the precise mechanism by
20   which any given compound functions to reduce methylation
in a microorganism, as measured by enhanced killing, is
not relevant to practicing the invention. Therefore,
effective methylation inhibitors, include those compounds
that inhibit methylation both directly and indirectly.
25   Many different enzymes and biochemical steps may be
inhibited, or otherwise altered to the detriment of the
microorganism or bacterium, as will be clear from the
detailed examples.

30   This invention is considered to be particularly
suitable for use in increasing the effectiveness of MLS
antibiotics against, e.g., gram-positive staphylococcus,
streptococcus, enterococcus and bacillus, gram-negative
cocci and gram-negative aerobes.
35

Bacteria that may thus be attacked include those
listed in Tables 6 and 7, and also, *Staphylococcus* spp.,

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S. sanguis, Corynebacterium diphtheria, Bacteroides spp.,
B. ovatus, Clostridium spp., *C. difficile, B. subtilis,*
Lactobacillus spp., *Campylobacter* spp., *Propionibacterium*
spp., *Mycoplasma* spp., *Fusobacterium, Corynebacterium,*
5   *Veillonella, S. fecalis, Nocardia farcinica,*
Actinobacillus actinomycetemcomitans, Group A and B
streptococci, Bacillus stearothermophilus, or *Pseudomonas*
aerugenosa.

10   The second agent inhibitors may thus be effectively
combined with one or more macrolide antibiotics, such as
erythromycin, azithromycin, clarithromycin,
roxithromycin, oleandomycin, spiramycin, josamycin,
miocamycin, midecamycin, rosaramycin, troleandomycin,
15   flurithromycin, rokitamycin or dirithromycin; one or more
lincosamide antibiotics, such as lincomycin, clindamycin,
celesticetin; or one or more streptogramin B antibiotics,
such as pristinamycin or virginiamycin. Erythromycin,
azithromycin, clarithromycin, lincomycin and clindamycin
20   are currently the most preferred.

        Suitable inhibitory second agents include those that
inhibit RNA methyltransferase enzymes, as described in
Example IV. These agents are exemplified by
25   S-adenosylhomocysteine (SAH) and analogues; homocysteine;
adenine derivatives, such as adenine phosphate, adenine
sulfate, adenine-N^1-oxide, 6-methyladenine,
6-mercaptoadenine, with dimethylallyladenine and
isopentenyladenosine being particularly preferred; and
30   SAM analogues and derivatives, such as methyl, ethyl,
n-propyl, allyl, n-butyl, n-pentyl, n-octyl and
6-amino-1-hexyl nitrogen analogues of SAM, and
S-inosyl-L-methionine, S-adenosyl-(5')-3-methylthio-
propylamine and 5'-methylthioadenosine.

35

        Further inhibitors of RNA methyltransferases include
sinefungin and analogues and metabolites, such as A9145C,

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5'-S-methylthioadenosine and cyclosinefungin;
nicotinamide and analogues, such as methylnicotinamide;
and polyinosinate. Specific examples of suitable
compounds include $S-N^6$-methyladenosylhomocysteine;

5   S-aristeromycinyl-L-homocysteine and analogues thereof,
such as 3-deazaaristeromycinyl-D-homocysteine;
5'-deoxy-5'-S-isobutyl-adenosine (SIBA) and ISOSIBA;
S-tubercidinylhomocysteine (STH) and analogues; MTA and
xylosyladenine.

10

It will be recognized that many compounds will exert
multiple effects on a microorganism or bacterial cell.
For example, SAH analogues and tubercidin act at various
points in methylation pathways. The division of the

15   useful agents into certain groups is thus not intended to
be an exact and non-overlapping scientific division.
Rather such information is set forth to assist those in
the art with understanding certain scientific data that
were considered by the inventors.

20

Second agents that inhibit S-adenosylhomocysteine
(SAH) hydrolase (SAHH) are also useful, as described in
Example V, and exemplified by adenosine and analogues.
Useful adenosine analogues include the first generation

25   agents 9(S)-(2,3-dihydroxypropyl)adenine [(S)-DHPA], D-
eritadine, (R,S)-3-adenine-9-yl-2-hydroxypropanocic acid
[(R,S)-AHPA], adenosine (Ado) dialdehyde, 3-
deazaadenosine (c^3-Ado), aristeromycin (Ari) and
neplanocin A (NPA or NpcA).

30

Second generation analogues are more preferred and
include dihydroxycyclopentenyladenine (DHCeA),
dihydroxycyclopentenyl-3-deazaadenine (c^3-DHCeA),
dihydroxycyclopentanyladenine (DHCaA),

35   dihydroxycyclopentanyl-3-deazaadenine (c^3-DHCaA),
3-deazaneplanocin A (c^3-NpcA), 3-deazaaristeromycin (c^3-
Ari), carbocyclic-3-deazaadenosine (C-c^3Ado), 6'-

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C-methylneplanocin A, 2'-deoxyadenosine and tubercidin.
Other useful analogues in this category include
ribavirin, pyraazofurin, 2'-deoxy-2'-chloroadenosine,
isopentenyladenosine and methylthioadenosine (MTA).

Other useful SAH hydrolase inhibitors include 9-β-
arabinofuranosyladenine (Ara-A, vidarabine) and
2'-Deoxyadenosine, of which Ara-A is already approved by
the FDA. Aristeromycin analogues, including
aristeromycin itself and N-methylaristeromycin, 8-
azaaristeromycin and 3-deazaaristeromycin, and their
dialdehyde and diol derivatives are also effective
inhibitors, as are (±)-5-Noraristeromycin and its 2,6-
diamino-analogue. Further useful aristeromycinyl
analogues with reduced toxicity include 2'-deoxy-,
3'-deoxy-, 3'-amino-3'-deoxy-, 3'-amino-
3'-deoxyarabinofuranosyl, 6'-hydroxy, 6'-mercapto,
8'-bromo, 8-hydroxyaristeromycin, aristeromycin-3'-cyclic
phosphate and aristeromycin-6'-cyclic phosphate, all of
which may be used in the present invention.

Certain structural analogues of SAH with
modification in the amino acid, base or sugar portion of
the molecule may be used to inhibit SAH hydrolase.
Useful analogues of this class include 2-fluoro-
S-adenosylhomocysteine (2-FSAH), S-Adenosyl-L-
homocysteine sulfoxide, S-Adenosyl-L-homocysteine
sulfone, S-aristeromycinyl-L-homocysteine, 5'-S-(3-
carboxyl-4-nitrophenyl)thioadenosine and 5'-S-(methyl)-
5'-S-(butyl)thioadenosine.

S-adenosylmethionine (SAM) synthetase (SAM) is
another target that may be inhibited, e.g., by using
second agents such as cycloleucine (1-aminocyclopentane-
1-carboxylic acid) and analogues; mono, bi- and
tri-cyclic amino acid structural and conformational
analogues of L-methionine; and ATP/methionine analogues,

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such as AMPNPP. Particular examples of cycloleucine and
methionine analogues include 1-aminocyclobutanecarboxylic
acid, 1-aminocyclohexanecarboxylic acid, L-2-amino-4-
hexanoic acid, (Z)-L-2-amino-5-chloro-trans-4-hexanoic
5   acid, L-ethionine, seleno-DL-ethionine, the (1R,2R,4S)
isomer of 2-aminonorbornane-2-carboxylic acid, the
(1R,2R,4S) isomer of 2-amino-5,6-exo-
trimethylenenorbornane-2-carboxylic acid,
3-aminobicyclo[3.2.0]heptane-3-carboxylic acid and,
10   preferably, (+)-2-aminobicyclo[2.1.1]hexane-2-carboxylic
acid.

       Further suitable inhibitors of (SAM) synthetase
include L-2-amino-4-methoxy-cis-but-3-enoic acid (L-cis-
15   AMB), L-2-amino-4-methylthio-cis-but-3-enoic acid (L-
cisAMTB) and covalent methionine-ATP adducts such as
5'(R)-(C)-[(L-homocysteine-S-yl)methyl]adenosine
5'-(β,γ)-imidotriphosphate.

20   Agents that inhibit, by any mechanism, the synthesis
of glutathione form another group of SAM inhibitors.
Inhibitors of glutathione synthetase are examples of
this class, and include buthionine sulfoximine,
7,8-dihydrofolate, α-aminomethylglutarate, SAPH-3 and
25   diethyl maleate. Methotrexate and trimethoprim, that
inhibit DHFR, also act to reduce glutathione synthetase
activity.

       Other agents that may be employed are methionine
30   synthetase (MS) and homocysteine transmethylase
inhibitors, such as nitrous oxide (N_2O), as commonly
employed in dentistry. Adenosine deaminase (ADA), and
even adenosine kinase, inhibitors may also be used. ADA
inhibitors include, e.g., coformycin, deoxycoformycin
35   (pentostatin) and isomers, 1,6-dihydro-6-hydroxy-
methylpurine nucleoside,
erythro-9-(2-hydroxy-3-nonyl)adenine, 6-methylaminopurine

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riboside and 2'-3'-isopropylidene-adenosine. Compounds
that inhibit the enzyme after ADA, purine nucleoside
phosphorylase, are also contemplated for use as they will
lead to a build up of inosine.

5

Dihydrofolate reductase (DHFR) inhibitors, such as
the clinically used drugs methotrexate (MTX), aminopterin
and trimethoprim, and 4,6-diamino-2,2-dimethyl-s-triazine
and 2,4-diamino-5-(3,4-dichlorophenyl)pyrimidine

10 analogues (e.g., NSC 127755 and 139105) may be used, as
may inhibitors of dihydropteroate synthetase, such as
sulfanilamide, sulfadiazine, sulfamethoxazole,
sulfisoxazole and sulfacetamide that also inhibit DHFR.

15 ^{5}N-Methyltetrahydrofolate (5-CH$_3$-FH$_4$) is used as a
methyl donor in the last step of methionine synthesis
from homocysteine in many bacterial reactions.
Inhibition of 5-CH$_3$-FH$_4$ synthesis will thus cause a
reduction in SAM synthesis. The inventors therefore

20 contemplate that agents that inhibit the production of
any of the biosynthetic precursors of 5-CH$_3$-FH$_4$, or those
that otherwise exert a negative influence on any of the
5-CH$_3$-FH$_4$ biosynthetic steps, will also be useful in the
present invention. Exemplary inhibitors are those listed

25 above such as MTX, NSC 127755, sulfonamides, and the
like.

Compounds that inhibit polyamine synthesis are also
useful. These include compounds such as

30 α-methylornithine, 1,3-diaminopropan-2-ol,
difluoromethylornithine (DFMO) and difluoromethylarginine
(DFMA). Also included are those compounds that inhibit
specific enzyme targets, such as ornithine decarboxylase
(ODC), arginine decarboxylase (ADC) and SAM Decarboxylase

35 (SAM-DC). Inhibitors of SAM-DC include AMA, MHZPA,
MAOEA, AbeAdo (MDL 73811), AdoMac, MGBG, CGP-39'937 and
CGP-33'829, as shown in Example X; inhibitors of ODC

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include eflornithine and other compounds described by
McCann & Pegg (1992) and Pegg & McCann (1992); and
inhibitors arginine decarboxylase include, by way of
example, DL-α-(difluoromethyl)arginine.

Compounds such as 5-azacitidine, cordycepin
(3'-deoxyadenosine), toyocamycin and analogues, that
inhibit RNA maturation, are also contemplated for use.
Antisense oligonucleotides may also be employed to
inhibit the induction of bacterial methylase synthetase
or as decoys that prevent the methylation of rRNA.
Antisense oligonucleotides may be advantageously
delivered using liposomes.

In the preparation of medicaments for the treatment
of animals or human patients, there are various
appropriate formulations and treatment regimens that may
be used. For example, the antimicrobial compound(s) and
second agent(s) may be administered to an animal
simultaneously, e.g., in the form of a single composition
that includes the microbial/antibiotic and second agent,
or by using at least two distinct compositions. The
antimicrobial agent could also be administered to the
animal prior to the second agent, although it is
currently more preferred to give the second agent
inhibitor prior to the antimicrobial agent.

The invention also provides novel compositions and
medicaments that contain a combination of antimicrobials
and second agents, not previously proposed for combined
use, dispersed in a pharmacologically acceptable
formulation. The antimicrobial agents and inhibitory
second agents may be formulated and administered in any
pharmacologically acceptable vehicle, such as parenteral,
topical, liposomal, nasal or ophthalmic preparations,
with formulations designed for oral administration being
currently preferred due to their ease of use.

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Multiple combinations may also be used, such as more than one antimicrobial agent used with one second agent or more than one second agent used with a given antimicrobial. Different classes of antimicrobial agents

5   and second agents may be combined, naturally following the general guidelines known in the art regarding drug interactions. Preferred combinations will be those in which inhibitors from different classes are used. Typically, between one and about five distinct

10   antimicrobial agents are contemplated for use along with between one and about ten distinct second agents. Combinations including at least one MLS antibiotic are currently preferred as there appears to be a particular need in the art for an improvement in MLS antibiotic

15   therapy.

Currently, one preferred group of inhibitory agents includes DHPA, D-eritadine, (R,S)-AHPA, adenosine dialdehyde, c^3-Ado, aristeromycin and neplanocin A; with

20   DHCeA, c^3-DHCeA, DHCaA, c^3-DHCaA, c^3-NpcA, c^3-Ari, C-c^3Ado, 6'-C-methylneplanocin A, tubercidin and Ara-A being more preferred. Further preferred compounds are polyinosinate, L-cis-AMB, L-cis-AMTB, buthionine sulfoximine, methotrexate, trimethoprim, N_2O, NSC 127755,

25   NSC 139105, DFMO, DFMA, coformycin, deoxycoformycin and sulfonamides.

Further embodiments of the invention include therapeutic kits that comprise, in suitable container

30   means, a pharmaceutical formulation of at least one antimicrobial agent and a pharmaceutical formulation of at least one microbial methylation inhibitor. The antibiotics and inhibitory second agents may be contained within a single container means, or a plurality of

35   distinct containers may be employed.

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BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present
specification and are included to further demonstrate
5   certain aspects of the present invention. The invention
may be better understood by reference to one or more of
these drawings in combination with the detailed
description of specific embodiments presented herein.

10

FIG. 1.   Erythromycin susceptibility for
Streptococcus aureus 6538 (erythromycin susceptible)
where ethionine, cycloleucine and trimethoprim are pre-
mixed with brain heart infusion (BHIA, filled bars) or
15   trypticase soya agar (TSA, unfilled bars).

FIG. 2.   Erythromycin susceptibility for *S. aureus*
27660 (erythromycin resistant), where ethionine (0.051M),
cycloleucine (0.10M) and trimethoprim (0.26mM) are pre-
20   mixed with BHIA (filled bars) or TSA (unfilled bars).

FIG. 3.   Erythromycin disc-susceptibility for *S.
aureus* 27660 (erythromycin resistant), pre-incubated with
inhibitor-containing discs. ((-----) denotes BHIA(E) and
25   (_____) denotes BHIA(E)$_c$)

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Antimicrobial combinations are well known and are
30   most frequently used to provide broad-spectrum empirical
coverage in the treatment of patients who are seriously
ill and who may be septicemic. Combinations are also
chosen because an identified pathogen is resistant to
inhibition and/or killing by conventional doses of a
35   single antibiotic, but in contrast is susceptible to the
combination (Eliopoulos & Moellering, 1991).

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Lorian (1991) indicated that there are generally four accepted mechanisms of antibacterial synergism, namely, (1) serial or sequential inhibition of a common biochemical pathway (e.g. trimethoprim-sulfamethoxazole);

5   (2) inhibition of protective bacterial enzyme (clavulanic acid plus a B-lactamase-susceptible penicillin); (3) combination of cell wall-active agents (e.g. amdincillin and ampicillin); and (4) use of cell wall-active agents to enhance the uptake of other antimicrobials (e.g.

10   penicillin and streptomycin). The present invention provides a novel synergistic option for antimicrobial treatment.

Antibiotic Resistance

15

Seven basic biochemical mechanisms for naturally-occurring antibiotic resistance have been described (Davies, 1986), namely alteration of the antibiotic; alteration of the target site; block in the transport of

20   the antibiotic; by-pass of the antibiotic sensitive-step; increasing the level of the inhibited enzyme; the cell is spared the antibiotic-sensitive step by endogenous or exogenous product; and the production of a metabolite that antagonizes action of inhibitor. The same general

25   concepts also apply to microorganisms other than bacteria.

Antibiotic and antimicrobial resistance can be spread vertically and horizontally by plasmids and

30   transposons. The inventors propose that methylation reactions play an active role in both plasmids and transposons, and that methylation inhibitors will modulate general antibiotic resistance. Butler & Gotschlich (1991) indicated that high-frequency

35   mobilization of broad-host-range plasmids into *Neisseria gonorrhoea* requires methylation in the donor. Additionally, they concluded that methylation/restriction

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systems was a major barrier against deficient conjugal transfer between *N. gonorrhoea* and heterologous hosts.

This invention therefore encompasses methods to
5   reduce antimicrobial resistance, caused by any of the seven mechanisms described above, using a combination of the antimicrobial agent and one or more inhibitor(s) that modify or influence methylation and the associated processes. A list of antibiotics that have developed
10 antibiotic resistance by one or more of these mechanisms is listed in Table 1 (taken from Lorian, 1991), all of which are intended to be combined with a methylation inhibitor, as described herein, thereby achieving enhanced microbial killing.
15

Table 2 (taken from Reese & Betts, 1993; Med. Let., 1992), lists the antibiotics generally preferred for use against a given pathogenic bacterium. It is contemplated that the effectiveness of all the antibiotics listed in
20 Table 2 will be increased upon combination with a methylation inhibitor. Table 3 (also taken from Reese & Betts, 1993), itemizes the common pathogenic bacteria that are implicated in focal infections. The present invention is thus contemplated for use against all such
25 infections.

TABLE 1

MECHANISMS OF RESISTANCE TO ANTIMICROBIAL AGENTS

Antimicrobial Agent	Mechanisms Causing Resistance	Examples of Organisms
Aminoglycosides	Modifying enzymes: acetyltransferases, adenylyl-transferases (nucleotidyl-transferases), phosphotransferases Ribosomal resistance (streptomycin, spectinomycin)	Enterobacteriaceae, P. aeruginosa, S. aureus, E. faecalis E. faecalis, Enterobacteriaceae, M. tuberculosis, P. aeruginosa
	Inadequate drug transport	E. faecalis, P. aeruginosa, anaerobes
β-lactams	Enzymatic inactivation	S. aureus, E. faecalis, Enterobacteriaceae, P. aeruginosa, Neisseria spp., H. influenzae
	Low affinity PBPs	S. pneumoniae, N. gonorrhoeae, S. aureus, P. aeruginosa
	Lack of penetration through outer membrane	P. aeruginosa, Enterobacteriaceae

TABLE 1 (continued)

Antimicrobial Agent	Mechanisms Causing Resistance	Examples of Organisms
Chloramphenicol	Acetylation	Enterobacteriaceae, S. aureus, streptococci, Bacteroides uniformis
	Lack of penetration	P. aeruginosa
Clindamycin, erythromycin, lincomycin	Ribosomal resistance due to methylation of rRNA	Streptococci, E. faecalis, Enterobacteriaceae
	Inactivation by esterase	Enterobacteriaceae
	Decreased penetration	S. hominis
Fluoroquinolones	Decreased uptake	Enterobacteriaceae, P. aeruginosa, staphylococci
	Altered target site (DNA gyrase)	Enterobacteriaceae, P. aeruginosa
Lincomycin	Inactivation	S. aureus
Sulfonamides	Synthesis of an altered or alternative target site (dihydropteroate synthetase)	Enterobacteriaceae, Neisseria spp., P. aeruginosa
	Lack of penetration	Anaerobes
	Overproduction of PABA	Neisseria, S. aureus

TABLE 1 (continued)

Antimicrobial Agent	Mechanisms Causing Resistance	Examples of Organisms
Tetracycline	Drug efflux	Enterobacteriaceae, staphylococci, streptococci
	Protection of ribosome from tetracycline	Streptococci, *E. faecalis*, *Neisseria* spp., *Mycoplasma* spp.
	Inactivation	Cryptic gene found in *B. fragilis*, expressed resistance in *E. coli*
Trimethoprim	Synthesis of an altered or alternative target site (dihydrofolate reductase)	Enterobacteriaceae, *V. cholerae*, staphylococci
	Lack of penetration	*P. aeruginosa*
	Ability to use alternative pathway	Enterococci
	Overproduction of dihydrofolate reductase	*H. influenzae*
Vancomycin	?	Pediococci, *Leuconostoc* spp. (intrinsic)
	?Blocking of target site	Enterococci (acquired)

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TABLE 2
ANTIBIOTICS OF CHOICE FOR COMMON PATHOGENS

Pathogen	Antibiotic of First Choice[a]	Alternative Agents[a]
Gram-positive cocci		
Staphylococcus aureus or *S. epidermidis* Non-penicillinase-producing	Penicillin	A first-generation cephalosporin, vancomycin, imipenem, or clindamycin; a fluoroquinolone[b]
Penicillinase-producing	Penicillinase-resistant penicillin (e.g., oxacillin or nafcillin)	A first-generation cephalosporin, vancomycin, clindamycin, imipenem, amoxicillin-clavulanic acid, ticarcillin-clavulanic acid, ampicillin-sulbactam; a fluoroquinolone[b]
Methicillin-resistant	Vancomycin with or without gentamicin and/or rifampin	TMP-SMZ, minocycline

TABLE 2 (continued)

Pathogen	Antibiotic of First Choice[a]	Alternative Agents[a]
Streptococci Group A, C, G	Penicillin	A cephalosporin[a], vancomycin, erythromycin; clarithromycin; azithromycin; clindamycin
Group B	Penicillin (or ampicillin)	A cephalosporin[a], vancomycin, or erythromycin
Enterococcus Endocarditis or other serious infection	Penicillin (or ampicillin) with gentamicin	Vancomycin with gentamicin
Uncomplicated urinary tract infection	Ampicillin or amoxicillin	A fluoroquinolone, nitrofurantoin
Viridans group	Penicillin G (with or without gentamicin)	A cephalosporin[a], vancomycin
S. bovis	Penicillin G	A cephalosporin[a], vancomycin
S. pneumoniae	Penicillin G	A cephalosporin[a], erythromycin, chloramphenicol, vancomycin

TABLE 2 (continued)

Pathogen	Antibiotic of First Choice[a]	Alternative Agents[a]
Gram-negative cocci		
Neisseria gonorrhoeae	Ceftriaxone	Spectinomycin, a fluoroquinolone, cefoxitin, cefixime, cefotaxime (see Appendix E)
N. meningitidis	Penicillin G	Third-generation cephalosporin, chloramphenicol
Moraxella (Branhamella) catarrhalis	TMP-SMZ	Amoxicillin-clavulanic acid; an erythromycin; clarithromycin azithromycin, cefuroxime, cefixime, third-generation cephalosporin, tetracycline
Gram-positive bacilli		
Clostridium perfringens (and Clostridium sp.)	Penicillin G	Chloramphenicol, metronidazole, or clindamycin
Listeria monocytogenes	Ampicillin with or without gentamicin	TMP-SMZ
Gram-negative bacilli		

TABLE 2 (continued)

Pathogen	Antibiotic of First Choice[a]	Alternative Agents[a]
Acinetobacter	Imipenem	Tobramycin, gentamicin, or amikacin, usually with ticarcillin or piperacillin (or similar agent); TMP-SMZ
Aeromonas hydrophila	TMP-SMZ	Gentamicin, tobramycin; imipenem; a fluoroquinolone
Bacteroides *Bacteroides* sp. (oropharyngeal)	Penicillin G	Clindamycin, cefoxitin, metronidazole, chloramphenicol, cefotetan, ampicillin-subactam
B. fragilis strains (gastrointestinal strains)	Metronidazole	Clindamycin; ampicillin-subactam; imipenem; cefoxitin[c]; cefotetan[c]; ticarcillin-clavulanic acid; piperacillin[c]; chloramphenicol; cefmetazole[c]
Campylobacter fetus, jejuni	A fluoroquinolone (adults) or an erythromycin	A tetracycline, gentamicin

TABLE 2 (continued)

Pathogen	Antibiotic of First Choice[a]	Alternative Agents[a]
Enterobacter sp.	Imipenem	An aminoglycoside and piperacillin or ticarcillin or mezlocillin; a third-generation cephalosporin[d]; TMP-SMZ; aztreonam; a fluoroquinolone
Escherichia coli Uncomplicated urinary tract infection	TMP-SMZ	A cephalosporin or a fluoroquinolone
Recurrent or systemic infection	A cephalosporin[e]	Ampicillin with or without an aminoglycoside, TMP-SMZ, oral fluoroquinolones useful in recurrent infections, ampicillin-sulbactam, ticarcillin-clavulanic acid, aztreonam

TABLE 2 (continued)

Pathogen	Antibiotic of First Choice[a]	Alternative Agents[a]
Haemophilus influenzae (coccobacillary) Life-threatening infections	Cefotaxime or ceftriaxone	Chloramphenicol; cefuroxime for pneumonia)
Upper respiratory infections and bronchitis	TMP-SMZ	Ampicillin or amoxicillin; cefuroxime; a sulfonamide with or without an erythromycin; cefuroxime-axetil; third-generation cephalosporin, amoxicillin-clavulanic acid, cefaclor, tetracycline; clarithromycin; azithromycin
Klebsiella pneumoniae	A cephalosporin[e]	An aminoglycoside, imipenem, TMP-SMZ, ticarcillin-clavulanic acid, ampicillin-sulbactam, aztreonam, a fluoroquinolone; amoxicillin-clavulanic acid
Legionella spp.	Erythromycin with rifampin	TMP-SMZ; clarithromycin; azithromycin; ciprofloxacin

TABLE 2 (continued)

Pathogen	Antibiotic of First Choice[a]	Alternative Agents[a]
Pasteurella multocida	Penicillin G	Tetracycline, cefuroxime, amoxicillin-clavulanic acid, ampicillin-sulbactam
Proteus sp.	Cefotaxime, ceftizoxime, or ceftriaxone[f]	An aminoglycoside; ticarcillin or piperacillin or mezlocillin; TMP-SMZ; amoxicillin-clavulanic acid; ticarcillin-clavulanic acid, ampicillin-sulbactam; a fluoroquinolone; aztreonam; imipenem
Providencia stuartii	Cefotaxime, ceftizoxime, or ceftriaxone[f]	Imipenem; an aminoglycoside often combined with ticarcillin or piperacillin or similar agent; ticarcillin-clavulanic acid; TMP-SMZ, a fluoroquinolone; aztreonam

TABLE 2 (continued)

Pathogen	Antibiotic of First Choice[a]	Alternative Agents[a]
Pseudomonas aeruginosa (nonurinary tract infection)	Gentamicin or tobramycin or amikacin (combined with ticarcillin, piperacillin, etc. for serious infections)	An aminoglycoside and ceftazidime; imipenem, or aztreonam plus an aminoglycoside; ciprofloxacin
(urinary tract infections)	Ciprofloxacin	Carbenicillin; ticarcillin, piperacillin, or mezlocillin; ceftazidime; imipenem; aztreonam; an aminoglycoside
Pseudomonas cepacia	TMP-SMZ	Ceftazidime, chloramphenicol
Salmonella typhi	Ceftriaxone	Ampicillin, amoxicillin, TMP-SMZ, chloramphenicol; a fluoroquinolone
Other species	Cefotaxime or ceftriaxone	Ampicillin or amoxicillin, TMP-SMZ, chloramphenicol; a fluoroquinolone
Serratia	Cefotaxime, ceftizoxime, or ceftriaxone[f]	Gentamicin or amikacin; imipenem; TMP-SMZ; ticarcillin, piperacillin, or mezlocillin; aztreonam; a fluoroquinolone
Shigella	A fluoroquinolone	TMP-SMZ; ceftriaxone; ampicillin

TABLE 2 (continued)

Pathogen	Antibiotic of First Choice[a]	Alternative Agents[a]
Vibrio cholerae (cholrea)	A tetracycline	TMP-SMZ; a fluoroquinolone
Vibrio vulnificus	A tetracycline	Cefotaxime
Xanthomonas (Pseudomonas) maltophilia	TMP-SMZ	Minocycline, ceftazidime, a fluoroquinolone
Yersinia enterocolitica	TMP-SMZ	A fluoroquinolone; an aminoglycoside; cefotaxime or ceftizoxime
Yersinia pestis (plague)	Streptomycin	A tetracycline; chloramphenicol; gentamicin

Key: TMP-SMZ = trimethoprim-sulfamethoxazole.
[a] Choice presumes susceptibility studies indicate that the pathogen is susceptible to the agent.
[b] The experience with fluoroquinolone use in staphylococcal infections is relatively limited. The fluoroquinolones should be used only in adults.
[c] Up to 15-20% of strains may be resistant.
[d] *Enterobacter spp.* may develop resistance to the cephalosporins.
[e] Specific choice will depend on susceptibility studies. Third-generation cephalosporins may be exquisitely active against many gram-negative bacilli (e.g., *E. coli, Klebsiella* sp.). In some geographic areas, 20-25% of community-acquired *E. coli* infections may be resistant to ampicillin (amoxicillin).
[f] In severely ill patients, this is often combined with an aminoglycoside while awaiting susceptibility data.

TABLE 3
COMMON PATHOGENS IN FOCAL INFECTIONS

Presumed location of infection	Common pathogens	Gram stain characteristics of exudate-if available
Urinary tract infections	Community-acquired: *Escherichia coli* Recurrent or nosocomial: *E. coli:* *Klebsiella, Proteus, Pseudomonas* sp. Enterococci	GNB GNB GPC
Intravenous catheter phlebitis and/or sepsis Peripheral catheter	*Staphylococcus aureus* or *S. epidermidis* *Klebsiella, Enterobacter, Pseudomonas* sp.	GPC GNB
Hyperalimentation line	*Candida* sp., *S. aureus, S. epidermidis*, enterococci *Klebsiella, Enterobacter* sp., etc.	Budding yeast; GPC GNB
Arteriovenous shunt	*S. aureus, S. epidermidis*	GPC
Septic bursitis	*S. aureus*	GPC

TABLE 3 (continued)

Presumed location of infection	Common pathogens	Gram stain characteristics of exudate-if available
Biliary tract	E. coli, Klebsiella sp., and enterococci; Bacteroides fragilis (in elderly patients), Clostridia sp.	
Intra-abdominal abscess, peritonitis, or large bowel perforation; diverticulitis[a]	E. coli B. fragilis Klebsiella sp. (Enterococci)	GNB GNB (thin, irregularly stained) GNB GPC
Burn wounds	Early: S. aureus, streptococci Later: gram-negative bacilli, fungi	
Cellulitis, wound and soft tissue infections	S. aureus Streptococci Clostridium sp.	GPC GPC GPB
Meningitis	See Appendix C	
Pneumonia	See Appendix D	

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TABLE 3 (continued)

Presumed location of infection	Common pathogens	Gram stain characteristics of exudate-if available
Pelvic abscess, postabortal or postpartal	Anaerobic streptococci B. fragilis Clostridium sp. E. coli Enterococci	GPC GNB (thin, irregularly stained) GPB GNB GPC
Septic arthritis	S. aureus Haemophilus influenzae (in children younger than 6 yr) Group B streptococci (in neonates)[b] Gram-negative organisms[b]	GPC GNC GPC GNB
Acute osteomyelitis	S. aureus H. influenzae (in children younger than 6 yr) Group B streptococci (in neonates)[b] Gram-negative organisms[b]	GPC GNC GPC GNB

Key: GNB = gram negative bacilli; GPC = gram-positive cocci; GPB = gram-positive bacilli; GNC = gram-negative coccobacilli.

[a]The precise role of enterococci in intra-abdominal infections is unclear. In mild to moderate infections, it may not be necessary to provide antibiotic activity against enterococci.

[b]In high-risk patients (e.g., immunocompromised, elderly, IV drug abusers, diabetics, debilitated patients).

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As a particular example, the β-lactams will be
described. To protect against the constantly changing
hostile environment, certain bacteria have developed a
cell membrane which includes complex carbohydrate-peptide
5   structures, assembled from individual carbohydrate and
amino acid monomers in the cytoplasm and then transported
out across the growing membrane for assembly. After
export and attachment to the growing cell membrane, a
free C-terminal end of a peptide is cross-linked to other
10   chains to give the required rigidity to the cell
membrane. Many β-lactam antibiotics block this step
which occurs on the outside of the membrane, and which
leads to weakening of the cell membrane and eventual
cell-lysis (Rapley & Walker, 1993).
15

To compensate, the bacterium produces lactamase
enzymes that hydrolyse an amide bond in the β-lactam
antibiotics to inactivate them. In order to achieve this
resistance, lactamase synthesis is induced in the
20   cytoplasm and this protein has to be exported to the
outer face of the membrane to be effective since the site
of β-lactamase antibiotics are on the outside of the
membrane. In gram positive bacteria, the β-lactamases
are targeted first to the periplasmic space by a
25   "peptide" leader sequence (Rapley & Walker, 1993).

As will now be indicated, many of the processes
described in this resistance mechanism are controlled by
methylation. In assessing this collection of diverse
30   information, the present inventors developed the idea
that antimicrobial resistance could be reduced in
practice using methylation inhibitors. Although many
scientific articles have analyzed the mechanisms
underlying antibiotic resistance, the idea of using
35   methylation inhibitors has not been proposed previously.

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The inventors now suggest that for the seven known antibiotic resistant mechanisms to operate, one prerequisite generally is the induction of the synthesis of at least one protein. They therefore predict that
5   methylase inhibitors will have the ability to influence most, if not all, of these seven mechanisms. Evidence available in the literature indicates that methylation inhibitors do influence these mechanisms, although there was no suggestion to use such inhibitors in a clinical
10 environment. It is now proposed that various methylation inhibitors alone could be used as antimicrobial agents and, more particularly, that their use in combination with known antimicrobials will reduce or eliminate resistance.
15

The present invention was developed in part from a detailed analysis of the scientific literature and an assimilation of known, but previously unconnected, facts. Certain of the publications in this area are described in
20 the following sections.

Butler & Gotschlich (1991) reported that antibiotic resistance in *Neisseria gonorrhoeae* had been associated with the acquisition of R plasmids from heterologous
25 organisms. RSF1010-like plasmids are the first multiresistant plasmid group found in Neisseria spp., having resistance not only to sulfonamides and streptomycin but also to ampicillin. They studied the possibility of dissemination of these plasmids into
30 *N. gonorrhoeae*. It was found that RSF1010 could be mobilized into *N. gonorrhoeae* at a very low frequency, but this frequency increases by greater than 4 orders of magnitude when the plasmid DNA is methylated *in vivo* by a specific methylase. It was suggested that protection
35 from digestion against the gonococcal restriction enzymes

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in vitro correlates with a substantial increase in the
conjugal frequency.

Stein *et al.* (1988) demonstrated that transformation
5   of gonococcal plasmid pFT180 isolated from *E. coli* HB101
or *N. gonorrhoeae* WR302 into that gonococcal strain PGH3-
2 required *in vitro* methylation with a specific
methylase. In the absence of methylation, a five-orders
of magnitude decrease in the transformation frequency was
10   observed.

Caboche & Bachellerie (1977) analyzed ribosomal RNA
maturation in eukaryotes and observed that the global
efficiency of the process of rRNA maturation is severely
15   affected in qualitative terms. The undermethylation
partially inhibits several stages in the maturation
pathway and therefore does not block selectively at a
definite step of processing. Two main alterations are
observed. First, in the nucleus the life times of the
20   various undermethylated intermediates of rRNA maturation
are markedly increased and a significant accumulation of
these forms is detected by long-term labelling studies.
Second, the rate of appearance of 32S rRNA into the
cytoplasm is severely affected in much higher proportions
25   than the formation of its immediate precursor, nuclear
28S rRNA, suggesting that the lack of methylation may
play an important role in the last step of maturation
(possibly release into the cytoplasm).

30   Although bacteria contain no nuclei, the processing
of rRNA is surprisingly similar to that of eukaryotes.
Additionally, fungi and parasites are eukaryotes and
should behave as described above. It is therefore
expected that cycloleucine should have some of the same
35   effects on bacterial rRNA maturation. The present
invention is thus contemplated to be useful against

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bacteria and other microbial infections, e.g., those
caused by yeast and parasitic microbes.

5   Yamaki et al. (1988) and others showed that dam
methylation is important in the regulation of initiation
of DNA replication in E. coli. These authors determined
that the thermal melting temperature of the oriC region
is lowered by adenine methylation at GATC sites. It is
proposed that dam methylation might lead to more
10   efficient transcription at promoters near oriC which may
stimulate initiation of DNA replication at oriC. Recent
studies also reportedly showed reduced frequency of
transformation of oriC plasmids in dam mutants, and poor
functioning of oriC plasmids derived from a dam mutant in
15   an in vitro DNA replication system. This leads the
inventors to predict that methylation inhibitors will
likely slow down the efficiency transcription and
replication of plasmids.

20   Sinefungin and SAH are known to effect DNA and
protein methylases from Streptomyces and other bacteria
and to inhibit the development of various fungi, viruses
and parasites (e.g., Barbes et al., (1990). It has been
reported that sinefungin inhibits the methylation of
25   adenine or guanine to a greater extent than cytosine in
tRNA (Vedel & Robert-Gero, 1981; Barbes et al., 1990).
It has also been observed that DNA methylases from
S. antibioticus, a non-sinefungin producer, are
noticeably inhibited, but not in the producer S.
30   incarnatus. Barbes et al. (1990) suggested that DNA
methylation, and possibly RNA methylation, appear to be
influenced in non-sinefungin producers. They also
suggested that sinefungin could be a useful tool in
investigating the physiological processes related to
35   bacterial methylation, but did not suggest the clinical

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use of sinefungin in combination with antibiotics to reduce antibiotic resistance.

Yebra *et al.* (1991) reported that sinefungin and derivatives inhibited RNA methyltransferase in *S. antibioticus, S. incarnatus* and *S. griseolus*. The structural features of sinefungin that are essential for its inhibitory effect were also identified (Yebra *et al.*, 1991, incorporated herein by reference). The maintenance of such important structural features in analogues intended for use in the present invention is required. When *E. coli* 16S and 23S rRNA were used as a substrate, lower exogenous methylation levels were observed, namely 10% of those obtained with tRNA.

Yebra *et al.* (1991) speculated further that, due to the instability of mRNA, that it is more conceivable that the modifications effected were on the precursors of mature rRNA and/or tRNA molecules during the process of formation. They also mentioned that sinefungin inhibits sporulation of *S. antibioticus*, but didn't inhibit the synthesis of DNA methyltransferases.

Lorian (1991) described the method of transduction in which bacteria acquire new DNA, including the transfer of resistance genes for β-lactamase and heavy metal-resistance plasmids of staphylococci, originally described in the 1960's and 1970's. These plasmids are packaged by a phage and carried from one cell to another, a process that has been shown to occur *in vitro* and *in vivo*.

It is generally accepted according to Lorian, that the most common mechanism of transfer of resistance genes is by conjugation in which cell-to-cell contact is made, and DNA from one cell (the donor) is transferred to

- 40 -

another cell (the recipient). Therefore, in the latter
case, the plasmid is able to mediate its own transfer by
conjugation.

5   Razin et al. (1975) showed that methylation of
bacteriophage DNA takes place during the last stage of
DNA replication. The inhibition of this DNA methylation
is accompanied by a corresponding decrease in phage
production, indicating the vital role of the methylation
10   step in the maturation of the virus. The Lorian and
Razin papers suggest to the inventors that methylation
inhibitors will likely disrupt plasmid/phage mediated
antibiotic resistance.

15   Zyskind et al. (1992) indicated that three cellular
processes occur during conversion of a mother bacterial
cell into daughter cells: DNA replication, cell growth,
and cell division. In procaryotic cells, the rate of
replication is controlled not by the rate of chain
20   elongation, but by the rate of initiation at the origin
of replication, oriC. The authors suggested that one or
more GATC sites in the origin region must be fully
methylated for the origin to function in initiation.
They concluded that as part of the mechanism, initiation
25   can occur only after methylation of the DNA of the oriC
region.

        Protein carboxymethylase is involved in mediating
bacterial chemotaxis (Diliberto et al., 1976; Aswad et
30   al., 1974). Flagellated bacteria require
S-adenosylmethionine's (SAM's) mediated methylation for
chemotaxis. Additionally, a rapid reversible methylation
of a protein in the cytoplasmic membrane of E. coli has
been reported to increase substantially during the
35   chemotactic response. The inventors expect that
methylation inhibition would influence these processes to

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the detriment of the organism and that the combination of methylation inhibitors and antimicrobial agents will likely be synergistic.

5   Park *et al.* (1987) showed that N-methylation of apocytochrome c facilitates the import of this protein into the mitochondria by a specific receptor based mechanism, which further illustrates the central role of methylation. This may be relevant when the present
10   invention is used against eukaryotic microbes.

         Pandit *et al.* (1992) indicated that the presence of methylated cytosine residues in the integrated DNA is strongly correlated with the number of plasmid copies.
15   They observed that *hph* genes heavily methylated contained many plasmid copies in transformants. In contrast, the genes those transformants that contains a single plasmid are not heavily methylated.

20   **Transmethylation**

         Most transmethylation reactions involve the transfer of a donor methyl group from SAM to an acceptor molecule and result also in the formation of
25   S-adenosylhomocysteine (SAH), which is an inhibitor of adenosylmethionine-dependent methyltransferases. Acceptor molecules in these reactions include protein carboxy groups, lysine groups, basic aminoacyl residues in proteins, phospholipids, nucleic acids and various
30   small molecules. These reactions play an essential role in specialized biological phenomena, such as chemotaxis, neurosecretion, membrane receptor interactions, DNA modification restriction, gene expression, DNA transcription, RNA localization, RNA processing and
35   cellular proliferation and differentiation (Lawrence & Robert-Gero, 1993).

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Methylation is known to be important in RNA
maturation Salim & Madden (1973). In HeLa cells, the 45S
precursor ribosomal RNA is about 13,000 nucleotides long
and is processed to form the 18S, 28S and 5.8S rRNAs.
5   Extensive spacer regions are eliminated during the
ribosomal RNA processing. Before processing, about 110
methylations, termed early methylation, occur rapidly in
the nucleolus on the 45S rRNA. These are 2'-O-ribose
methylations, and they occur in molar yields for nearly
10   all positions. Cleavage then occurs to produce smaller
RNAs in which all of the methyl groups are conserved.
The 45S goes to 41S which, in turn, splits into two
pathways: first, 41S to 32S to 28S rRNA; and second, 41S
to 20S to 18S rRNA. Subsequently, during late
15   maturation, seven base methylations occur.

It is known that inhibition of methylation leads to
serious extensive degradation of rRNA in Hela cells
(Lhoest & Colson, 1990). Although these authors cited
20   studies where it appears that under methylation does not
play an important role in prokaryotic rRNA maturation,
other studies showed that inhibition of methylation by
sinefungin in S.cerevisiae leads to a decrease in stable
18S rRNA (Li et al., 1985).
25

SAM serves as the methyl donor for numerous
methyltransferase reactions (De Clercq, 1987). Effective
methylation requires the concerted action of a number of
enzymes: methyltransferases, SAH hydrolase,
30   dihydropteroate synthetase, dihydrofolate reductase, SAM
synthetase, methionine synthetase, homocysteine
transmethylase, ornithine decarboxylase, arginine
decarboxylase and adenosine deaminase.

35   SAM itself is synthesized from ATP and methionine.
SAH, which is a product of, and an inhibitor of,

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methyltransferase reactions, is generated when transfer
of a methyl group from SAM to acceptor substrate occurs.
To avoid SAH-mediated of inhibition and to allow the
methyltransferases to proceed with their action, SAH has

5   to be removed by the enzyme SAH hydrolase. SAH hydrolase
catalyzes the reversible hydrolysis of SAH to
homocysteine and adenosine. Homocysteine and adenosine,
in turn, are product inhibitors of the SAH hydrolase
reaction, which means that they have to be removed if SAH

10   hydrolase is to proceed with its catalatic function.
This can be achieved by several pathways which involve
conversion of homocysteine to methionine, to cysteine or
to oxidation products, and conversion of adenosine to
inosine by adenosine deaminase.

15

It can thus be seen that effective methylation
requires the concerted action of at least three types of
enzymes: methyltransferases, SAH hydrolase, adenosine
deaminase and several enzymes involving homocysteine.

20   Based on this requirement, the inventors envision certain
primary types of methylation inhibitors for use in
connection with MLS antibiotics: compounds that inhibit
methyltransferases; SAH analogues, acyclic and
carbocyclic adenosine analogues that inhibit SAH

25   hydrolase; inhibitors of adenosine deaminase and
homocysteine metabolism.

Furthermore, methylation will also be influenced if
the synthesis of SAM or methionine is altered.

30   Therefore, inhibition of any enzymes required for the
synthesis of SAM, such as SAM synthetase, dihydropteroate
synthase, dihydrofolic reductase and
homocysteinetransmethylase are contemplated to be
effective ways by which to lower the effective

35   concentration of SAM, and thus to decrease the level of
methylation reactions. Additionally, compounds that

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inhibit maturation of rRNA subunits and any step in protein translation are also contemplated to be useful, as are compounds that inhibit polyamine synthesis.

5   A large number of methylation inhibitors have been described in the literature and, although none have been proposed for use in conjunction with reducing MLS antibiotic resistance, many such inhibitors have been studied for their ability to affect mRNA and small
10   molecule methylation. The preparation and use of various compounds and agents that are capable of inhibiting bacterial RNA methylation, synthesis and/or maturation is described further in the detailed examples.

15   **MLS Antibiotics**

Weisblum (1984) reported that what appeared initially as uncomplicated resistance to erythromycin in gram positive bacteria, actually constituted a resistance
20   syndrome, dependent upon biochemical modification of the 50S ribosome subunit, which in turn, confers resistance to three chemically distinct classes of antibiotics - the macrolides, lincosamides, and streptogramin type B (MLS) antibiotics. Bacteria become resistant to erythromycin
25   by single biochemical alteration of the ribosomes, as a result of which, the affinity between the drug and ribosome is reduced. By this single alteration, namely, N^6-methylation or N^6,N^6-dimethylation of adenine in 23S ribosomal RNA, a structural component of the 50S ribosome
30   subunit, bacteria become more generally resistant to the MLS antibiotics.

The MLS antibiotics comprise three of at least ten chemical distinct classes that inhibit protein synthesis
35   by their action on the 50S ribosomal subunit. In view of the apparent functional relationship defined by observed

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co-resistant patterns, these three group of inhibitors
were designated collectively as the MLS antibiotics.

MLS antibiotics are chemically distinct but have a
5   similar mode of action. Their spectrum of activity
includes gram-positive cocci (in particular,
staphylococci, streptococci, and enterococci) and bacilli
and gram negative cocci. These drugs, especially
clindamycin, are also potent against anaerobic bacteria.
10   Gram-negative bacilli are usually resistant to MLS
antibiotics, but certain enteric bacilli and *Haemophilus*
spp. are susceptible to azithromycin. The activities of
these antibiotics against *Campylobacter, Legionella* and
Chlamydia spp. are at the origin of the recent
15   renaissance of erythromycin.

Macrolides are composed of a minimum of two amino
and/or neutral sugars attached to a lactone ring of
variable size. Macrolides that are commercially
20   available or in clinical development can be divided into
14-, 15-. and 16-membered lactone ring macrolides. These
classes differ in their pharmacokinetics properties and
in their responses to bacterial resistance mechanisms.
Lincosamides (lincomycin and the more active
25   semisynthetic derivative clindamycin) are alkyl
derivatives of proline and are devoid of a lactone ring.
Streptogramin antibiotics are used clinically in certain
countries, including Belgium and France. They are
composed of two factors, A and B (I and II in
30   pristinamycin and M and S in virginiamycin,
respectively), that act synergistically and are produced
by the same microorganism.

In 1956, a few years after the clinical introduction
35   of erythromycin, resistance of staphylococci to this drug
emerged and subsequently spread to France, the United

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Kingdom, and the United States. The MLS cross-resistance
phenotype due to modification of the drug rRNA target is
widely distributed. Resistance has since been detected
in *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus*
5   spp., *Corynebacterium diphtheria*, *Bacteroides* spp.,
Clostridium spp., *Bacillus* spp., *Lactobacillus* spp.,
Mycoplasma pneumonia, *Campylobacter* spp.
Propionibacterium spp. and members of the family
Enterobacteriaceae. (LeClercq & Courvalin, 1991). The
10   structure of erythromycin A, the proto-typical macrolide
is shown below:
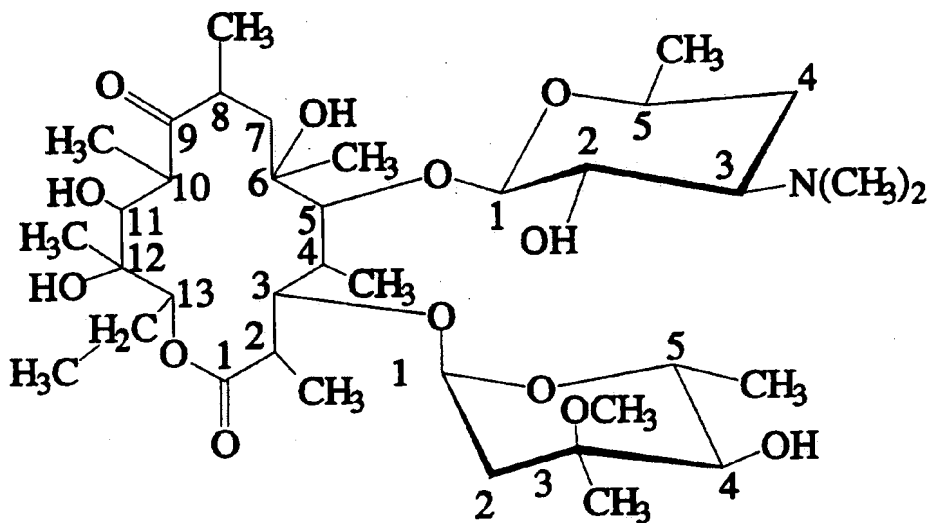
In the erythromycin family, three compounds are
typically used clinically: erythromycin, azithromycin
15   and clarithromycin (Reese & Betts, 1993). Erythromycin
is one of the safest antibiotics in current clinical use.
Bacteria known to be susceptible to erythromycin are
listed in Table 6 (taken from Lorian, 1991) and in Table
7 (taken from Reese & Betts, 1993). This further serves
20   to indicate the utility of the invention and the wide-

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ranging clinical use for the combinations disclosed herein.

5   Azithromycin a new azolide antibiotic approved for clinical use in 1991. This is a novel 15-member azolide antibiotic. Its structure differs from that of erythromycin in that the lactone ring contains a nitrogen atom. This molecular rearrangement has resulted in a compound with an increased spectrum of activity, yielding

10   high and sustained tissue antibiotic levels that are much greater than the serum antibiotic levels, and prolonged tissue half-life. This allows for fewer doses during a course of therapy.

15   Clarithromycin is chemically different from erythromycin by having an O-methyl substitution at position 6 of the macrolide ring. Its spectrum of activity is similar to that of erythromycin, except for enhanced *H. influenzae* activity, but it has better

20   stability properties, including a twice-daily dose regimen.

       The mechanism of action of the erythromycin-like antibiotics involves binding to rRNA and prevention of

25   bacterial protein synthesis. The ermC gene confers resistance to erythromycin in gram-positive bacteria by specifying a 23S rRNA methylase that N^6-methylates or N^6,N^6-dimethylates an adenine residue at position 2,058 on the 23S rRNA, thus decreasing ribosomal affinity for

30   erythromycin at that site (Skinner *et al.*, 1983; Weisblum *et al.*, 1971). The ermC expression is induced by erythromycin, and erythromycin binding to unmethylated ribosomes is required for induction. The induction mechanism, termed translational attenuation, has been

35   intensively studied (Denoya *et al.*, 1986).

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    The ermC mRNA exists in an inactive confirmation in
which the ribosome binding site (Shine Dalgarno sequence
[CD2]) for methylase synthesis is sequestered by base
pairing in a double-stranded region. Stalling of a
5   ribosome under the influence of erythromycin binding
during translation of a 19 amino acid leader peptide
causes the inactive mRNA structure to open, allowing
initiation of methylase synthesis. Translation induction
appears to be further regulated by a methylation-mediated
10   feedback loop. Upon induction, the intracellular level
of methylase increases, as does the number of methylated
ribosomes, with a co-commitment decrease of the ribosomal
affinity for erythromycin and prevention of further
induction. The overall regulation of ermC expression has
15   been summarized by Denoya et al. (1986).

    Zalacain & Cundliffe (1990) described methylation of
23S ribosomal RNA due to carB, an antibiotic-resistance
determinant from the carbomycin producer Streptomyces
20   thermotolerans. They showed the carB product
monomethylates the amino group of the adenosine residue
located at position 2058 in 23S rRNA. In contrast,
ribosomes from S. lividans expressing ermE, in which the
23S rRNA is dimethylated at this same position, are
25   highly resistant to macrolides and insensitive to
lincosamides. Thakker-Varia et al. (1985) reported that
the methylation reaction is affected by a class of
methylase, whose genes are typically plasmid- or
transposon-associated, and whose synthesis is inducible
30   by erythromycin.

    There are apparently no practical proposals in the
literature as to how bacterial resistance to MLS
antibiotics may be overcome. Although generally
35   applicable to all antimicrobial agents, the present
inventors particularly propose the use of one or more

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methylation inhibitors, in conjunction with MLS
antibiotics. These will reduce the bacterium's ability
to methylate rRNA and allow the antibiotics to bind to
their natural rRNA targets, thus inhibiting protein

5   synthesis. Although elegantly simple in hindsight, this
represents a novel and important development in
anti-microbial strategies.

       The following examples are included to demonstrate
10   preferred embodiments of the invention. It should be
appreciated by those of skill in the art that the
techniques disclosed in the examples that follow
represent techniques proposed by the inventors to
function well in the practice of the invention, and thus
15   can be considered to constitute preferred modes for its
practice. However, those of skill in the art should, in
light of the present disclosure, appreciate that many
changes can be made in the specific embodiments that are
disclosed and still obtain a like or similar result
20   without departing from the spirit and scope of the
invention.

EXAMPLE I

Antibiotic Treatment

25

       Antibiotics such as erythromycin, clindamycin,
azithromycin, clarithromycin, vancomycin and the like,
may be used clinically at a range of doses. Depending on
the circumstances, antimicrobial agents may be employed
30   in oral or parenteral treatment regimens. Appropriate
doses are well known to those of skill in the art and are
described in various publications, such as (Reese &
Betts, 1993; incorporated herein by reference). Table 4
and Table 5 (taken from Reese & Betts, 1993) are included
35   herein to provide ready reference to the currently
recommended doses of a variety of antimicrobial agents.

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Following are definitions of terms that are used in Table 4 and Table 5:   qid (4 times daily), tid (3 times daily), bid (twice daily), qd (once daily), q4h (every 4 hours around the clock), q6h (every 6 hours around the clock) and q8h (every 8 hours around the clock).

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TABLE 4. COMMON ANTIBIOTICS AND USUAL ORAL DOSES

ANTIBIOTIC	DOSAGE

Penicillin V
    Rugby (generic)
    V-cillin K
	250 mg qid

Dicloxacillin
    Glenlawn (generic)
    Dynapen
	250 mg qid

Cloxacillin (Tegopen)	250 mg qid

Amoxicillin
    Rugby (generic)
    Polymox
	250 mg tid

Ampicillin
    Moore (generic)
    Polycillin
	250 mg qid

Augmentin
    250-mg tablets
    chewables (250 mg)
    125-mg (suspension)
    chewables (125 mg)
	tid

Carbenicillin (Geocillin)	382 mg qid (1 tb) 2 tab qid

Cephalexin
    Rugby (generic)
    Keflex
    Rugby (generic)
    Keflex
	250 mg qid
	
	500 mg qid

Cefadroxil
    Rugby (generic)
    Duricef
	1 gm bid

Cephradine
    Rugby (generic
    Velosef
    Rugby (generic)
    Velosef
	250 mg qid
	
	500 mg qid

Cefaclor Ceclor	250 mg tid
Cefuroxime axetil Ceftin	125 mg bid 250 mg bid 500 mg bid
Cefixime Suprax	400 mg q24h

TABLE 4 (continued)   - 52 -

ANTIBIOTIC	DOSAGE
Cefprozil Cefzil	250 mg q12h
Loracarbef (Lorabid)	200 mg bid
Cefpodoxime proxetil (Vantin)	200 mg bid
Clindamycin Cleocin	300 mg q8h

TMP/SMZ
  Bactrim
  Septra
  (generic)
	1 double-
	strength bid

Trimethoprim
  Rugby (generic)
  Proloprim
	100 mg bid

Erythromycin (base)
  Abbott
  E-mycin (delayed release)
	250 mg qid

Erythromycin stearate Rugby (generic)	250 mg qid
Azithromycin Zithromax	1 g once only 500 mg, day 1, plus 250 mg, day 2-5
Clarithromycin Biaxin	250 mg bid 500 mg bid

Tetracycline hydrochloride
  Mylan
  Sumycin 250
	250 mg qid

Doxycycline
 	
  Lederle (generic)
  Vibramycin
	100 mg qd (with
	200-mg initial
	load)

Vancomycin Vancocin HCl (oral soln/powder)	Capsules 125 mg q6h PO

Metronidazole
  Rugby (generic)
  Flagyl
	250 mg qid

Norfloxacin Noroxin	400 mg bid

TABLE 4 (continued)   - 53 -

ANTIBIOTIC	DOSAGE
Ciprofloxacin Cipro	250 mg bid 500 mg bid 750 mg bid
Ofloxacin Floxin	200 mg bid 300 mg bid 400 mg bid
Lomefloxacin Maxaquin	400 mg once qd

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TABLE 5.   COMMON ANTIBIOTICS AND USUAL PARENTERAL DOSES

ANTIBIOTIC	DOSAGE
Penicillin G Pfizerpen G (Pfizer)	2,400,000 units 12 million units
Oxacillin Prostaphlin (Bristol)	12 g
Nafcillin Nafcil (Bristol)	12 g
Ampicillin Omnipen (Wyeth)	6 g
Ticarcillin Ticar (Beecham)	18 g
Piperacillin Pipracil (Lederle)	18 g 16 g
Mezlocillin Mezlin (Miles)	18 g 16 g
Ticarcillin-clavulanate Timentin (Beecham)	18 g/0.6 g 12 g/0.4 g
Ampicillin-sulbactam Unasyn (Roerig)	6 g 12 g
Cephalothin Keflin (Lilly)	9 g (1.5 g q4h)
Cefazolin Ancef (SKF)	4 g (1 g q6h) 3 g (1 g q8h)
Cefuroxime Zinacef (Glaxo)	6 g2.25 g (750 mg q8h) 4.5 g (1.5 g q8h)
Cefamandole Mandol (Lilly)	9 g (1.5 g q4h)
Cefoxitin Mefoxin (MSD)	8 g (2 g q6h) 6 g (2 g q8h)
Cefonicid Monicid (SKF)	1 g q12h
Cefotetan Cefotan (Stuart)	2 g q12h
Cefmetazole Zefazone (Upjohn)	2 g q8h
Ceftriaxone Rocephin (Roche)	2 g (2.0 g q24h) 1 g (1.0 g q24h)

TABLE 5 (continued)   - 55 -

ANTIBIOTIC	DOSAGE

Ceftazidime
    Fortax (Glaxo)
    Taxicef (SKF)
    Tozidime (Lilly)
	6 g (2 g q8h)

Cefotaxime Claforan (Hoechst)	2 g q6h 2 g q8h
Cefoperazone Cefobid (Pfizer)	8 g (2 g q6h) 6 g (2 g q8h)
Ceftizoxime Ceftizox (SKF)	(2 g q8h)
Aztreonam Azactam (Squibb)	2 g q8h 1 g q8h
Imipenem Primaxin (MSD)	2000 mg (500 mg 16h)

Gentamicin
  Garamycin
    (Schering)
  (generic) (Elkins-Sinn)
	360 mg (1.5 mg/kg q8h for an 80-kg patient)

Tobramycin Nebcin (Dista)	360 mg (1.5 mg/kg q8h for an 80-kg patient)
Amikacin Amikin (Bristol)	1200 mg (7.5 mg/kg q12h for an 80-kg patient)
Clindamycin Cleocin (Upjohn)	2400 mg (600 mg q6h) 2700 mg (900 mg q8h) 1800 mg (600 mg q8h)
Chloramphenicol Chloromycetin (P/D)	4 g (1 g q6h)
TMP/SMZ Septra (Burroughs Wellcom)	1400 mg TMP (5 mg TMP/kg q6h for a 70-kg patient) 700 mg TMP (5 mg TMP/kg q12h for a 70-kg patient)

Erythromycin
    Erythromycin
    (Elkins-Sinn)
	2000 mg (500 mg q6h)

TABLE 5 (continued)   - 56 -

ANTIBIOTIC	DOSAGE
Doxycycline Vibramycin (Pfizer)	200 mg (100 mg q12h)
Vancomycin Vancocin (Lilly)	2000 mg (500 mg q6h)
Metronidazole (generic) (Elkins-Sinn)	2000 mg (500 mg q6h)
Ciprofloxacin Cipro	200 mg q12h 400 mg q12h
Pentamidine Pentam (LyphoMed)	280 mg (4 mg/kg q24h for a 70-kg patient)

The effectiveness of erythromycin and lincomycin against a wide variety of organisms is shown in Table 6 (taken from Lorian, 1991). Table 7 (taken from Reese & Betts, 1993), is provided as a comparison of certain common MLS antibiotics. The data presented in the tables of the present specification is another tool to enable the straightforward comparison of raw data with accepted clinical practice and to allow the determination of appropriate doses of combined agents for clinical use.

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TABLE 6
SUSCEPTIBILITY TO ANTIBIOTICS

ERYTHROMYCIN

Species	(n)	Range	MIC$_{50}$	MIC$_{90}$
Bacillus spp.	20	0.03-2	0.25	2
Bacteroides fragilis	97	≤0.25-16	1	8
Bordetella bronchiseptica	11	4-32	8	32
Bordetella parapertussis	46	≤0.125-4	0.25	0.25
Bordetella pertussis	32	1-0.5	0.25	0.25
Bordetella pertussis	75	≤0.125-0.5	≤0.125	≤0.125
Borrelia burgdorferi	10	0.03-0.125	0.03	0.06
Branhamella (Moraxella) catarrhalis	20	≤0.125-0.5	0.25	0.25
Branhamella (Moraxella) catarrhalis	20	0.125-0.5	0.25	1
Branhamella (Moraxella) catarrhalis (non β-lactamase producer)	40	≤0.06-0.5	0.25	0.5
Branhamella (Moraxella) catarrhalis (non β-lactamase producer)	13	0.03-0.125	0.06	0.06
Branhamella (Moraxella) catarrhalis (non β-lactamase producer)	14	≤0.06-1	0.125	1
Branhamella (Moraxella) catarrhalis (non β-lactamase producer)	16	0.015-1	0.06	0.25

TABLE 6 (continued) — 58 —

Species	(n)	Range	MIC$_{50}$	MIC$_{90}$
Branhamella (Moraxella) catarrhalis (β-lactamase producer)	47	≤0.06-1	0.25	0.5
Branhamella (Moraxella) catarrhalis (β-lactamase producer)	58	0.03-0.25	0.125	0.125
Branhamella (Moraxella) catarrhalis (β-lactamase producer)	160	≤0.06-8	0.25	0.5
Branhamella (Moraxella) catarrhalis (β-lactamase producer)	35	0.03-0.125	0.06	0.06
Campylobacter jejuni	25	0.5-8	1	4
Campylobacter jejuni	16	0.125-4	0.25	2
Campylobacter pylori	56	0.25-16	0.5	1
Campylobacter pylori	13	0.125-0.25	0.125	0.25
Corynebacterium JK	102	0.5-≥128	≥128	≥128
Corynebacterium JK	19	0.125-≥64	2	≥64
Enterococcus faecalis	26	1-≥64	1	4
Enterococcus faecalis	50	0.06-≥64	4	≥64
Enterococcus faecalis	86	0.125-≥64	1	≥64
Enterococcus faecalis	97	0.125-128	2	128
Enterococcus faecium	14	≤0.06-≥64	1	≥64
Enterococcus spp.	35	≤0.06-≥32	2	≥32
Haemophilus ducreyi	122	?-0.125	0.004	0.06
Haemophilus influenzae	145	0.5-8	2	2

TABLE 6 (continued)   - 59 -

Species	(n)	Range	MIC$_{50}$	MIC$_{90}$
Haemophilus influenzae	97	0.25-≥16	1	4
Haemophilus influenzae (non β-lactamase producer)	22	0.125-8	2	4
Haemophilus influenzae (non β-lactamase producer)	137	≤0.06-≥8	4	8
Haemophilus influenzae (β-lactamase producer)	46	≤0.06-≤8	4	8
Haemophilus influenzae (β-lactamase producer)	17	0.25-4	2	4
Haemophilus influenzae (penicillin susceptible)	22	0.25-16	8	16
Haemophilus influenzae (penicillin resistant)	20	8-16	8	16
Haemophilus parainfluenzae	13	0.5-8	2	4
Legionella spp.	23	0.03-0.25	0.125	0.25
Legionella pneumophila	31	≤0.0075-0.25	0.06	0.125
Legionella pneumophila	48	0.03-2	0.25	0.5
Legionella pneumophila	25	0.125-1	0.25	1
Listeria monocytogenes	13	0.5-1	0.5	0.5
Listeria monocytogenes	16	0.125-2	0.25	1
Listeria monocytogenes	65	≤0.06-≥32	0.125	32

TABLE 6 (continued)   - 60 -

Species	(n)	Range	MIC_{50}	MIC_{90}
Mycoplasma hominis	26	≥128	≥128	≥128
Mycoplasma hominis	20	≥256	≥256	≥256
Mycoplasma pneumoniae	10	≤0.06-8	≤0.06	≤0.06
Mycoplasma pneumoniae	14	≤0.004-0.03	≤0.004	≤0.004
Neisseria gonorrhoeae	19	0.0075-8	0.25	1
Neisseria gonorrhoeae (non β-lactamase producer)	73	0.015-4	0.25	2
Neisseria gonorrhoeae (non β-lactamase producer)	78	0.03-2	0.25	1
Neisseria gonorrhoeae (β-lactamase producer)	12	0.03-4	0.5	2
Neisseria gonorrhoeae (β-lactamase producer)	17	1-4	2	4
Neisseria meningitidis	19	0.5-8	1	8
Nocardia asteroides	78	≤0.25-≥8	≥8	≥8
Staphylococcus aureus	44	0.125-1	0.125	0.5
Staphylococcus aureus	100	0.25-128	0.5	4
Staphylococcus aureus (penicillin susceptible)	20	0.125-0.5	0.5	0.5
Staphylococcus aureus (penicillin susceptible)	35	≤0.06-≥32	0.25	0.5

TABLE 6 (continued)   - 61 -

Species	(n)	Range	MIC$_{50}$	MIC$_{90}$
Staphylococcus aureus (penicillin resistant)	35	0.25-≥32	0.25	≥32
Staphylococcus aureus (methicillin susceptible)	28	0.125-1	0.25	0.5
Staphylococcus aureus (methicillin susceptible)	97	≤0.125-≥64	0.25	≥64
Staphylococcus aureus (methicillin susceptible)	20	0.125-1	0.5	0.5
Staphylococcus aureus (methicillin resistant)	17	0.5-≥128	128	128
Staphylococcus aureus (methicillin resistant)	15	≥64	≥64	≥64
Staphylococcus aureus (methicillin resistant)	20	≥64	≥64	≥64
Staphylococcus aureus (methicillin resistant)	30	≤0.06-≥32	≥32	≥32
Staphylococcus coagulase φ	10	0.125-4	0.25	2
Staphylococcus coagulase φ	100	0.125-≥64	0.25	≥64
Staphylococcus coagulase φ (non β-lactamase producer)	12	0.03-8	0.125	0.25
Staphylococcus coagulase φ (β-lactamase producer)	38	0.06-16	0.125	4

TABLE 6 (continued)   - 62 -

Species	(n)	Range	MIC$_{50}$	MIC$_{90}$
Staphylococcus epidermidis	50	0.125-≥64	≥64	≥64
Staphylococcus haemolyticus	20	0.125-≥64	≥64	≥64
Staphylococcus hominis	20	0.125-≥64	≥64	≥64
Streptococcus agalactiae	20	0.03-0.25	0.03	0.125
Streptococcus agalactiae	34	0.015-0.06	0.03	0.03
Streptococcus pneumoniae	58	0.03-0.25	0.06	0.125
Streptococcus pneumoniae	91	0.125-4	0.125	0.125
Streptococcus pneumoniae	50	0.015-0.06	0.03	0.03
Streptococcus pneumoniae	16	≤0.03-0.125	0.06	0.125
Streptococcus pneumoniae	26	0.015-0.25	0.03	0.06
Streptococcus pneumoniae	50	0.03-0.125	0.06	0.06
Streptococcus pyogenes	19	0.03-0.25	0.06	0.125
Streptococcus pyogenes	20	≤0.03-0.25	0.06	0.125
Streptococcus pyogenes	33	0.015-0.03	0.03	0.03
Streptococcus pyogenes	20	≤0.06-≥32	0.125	≥32
Streptococcus spp.	22	0.015-0.25	0.03	0.06
Streptococcus spp.	107	0.004-2	0.03	1
Ureaplasma urealyticum	28	0.015-≥256	2	≥256
Ureaplasma urealyticum	19	8-≥128	16	32

TABLE 6 (continued)   - 63 -

LINCOMYCIN

Species	(n)	Range	MIC_{50}	MIC_{90}
Mycoplasma hominis	28	0.5-16	2	4
Mycoplasma pneumoniae	11	2-32	8	32
Staphylococcus aureus	100	0.5-512	1	1
Ureaplasma urealyticum	19	64-≥128	≥128	≥128

TABLE 7. *IN VITRO* ACTIVITY AND COMPARISON OF SELECTED MACROLIDE ANTIBIOTICS

Organism	Azithromycin MIC$_{90}$	Clarithromycin MIC$_{90}$	Erythromycin MIC$_{90}$
Gram-positive aerobes			
Staphylococcus aureus			
Methicillin susceptible	1.0[a]	0.12-0.24[a]	0.25-0.50
Methicillin resistant	>128.0	>128.0	>128.0
Streptococcus pyogenes (group A)			
Erythromycin susceptible	0.12[a]	0.015[a]	0.03[a]
Streptococcus pneumoniae	0.12[a]	0.015[a]	0.03-1.0[a]
Streptococcus agalactactiae (group B)	0.5[a]	0.03-0.25[a]	0.03-0.25[a]
Streptococcus bovis	0.25		
Enterococci (group D)			
E. faecalis			
(Erythromycin susceptible)	8.0		
(Erythromycin resistant)	>64.0		
E. facium			
Viridans streptococci	16.0	0.03	0.06
Coagulase-negative staphylococci			
Methicillin resistant	>128.0		
Methicillin susceptible			
Listeria monocytogenes	4.0	0.12-2.0	0.5-2.0

TABLE 7 (continued)

Organism	Azithromycin MIC$_{90}$	Clarithromycin MIC$_{90}$	Erythromycin MIC$_{90}$
Gram-negative aerobes			
Haemophilus influenzae	1.0	2.0-16.0	4.0-8.0
Moraxella catarrhalis	0.5	0.25-1.0	0.25-2.0
Neisseria gonorrhoeae	0.15	0.25-2.0	0.25-2.0
N. meningitidis	0.12	--	0.4-1.6
Pasteurella multocida	0.5	--	--
Bordetella pertussis	0.12	--	0.3-1.6
Legionella pneumophila	0.25-2.0	0.25	1.0-2.0
Campylobacter species	0.25	1.0-8.0	0.2->50.0
Anaerobes			
Gram-positive cocci (peptococcus, peptostreptococcus)	2.0	4.0->32.0	2.0->32.0
Bacteroides fragilis	8.0	2.0-8.0	4.0-32.0
Clostridium perfringens	1.0	0.5-2.0	1.0
Miscellaneous			
Chlamydia pneumoniae (formerly TWAR)	0.12-0.25-1.0	0.007	0.065-1.0
Chlamydia trachomatis	0.12-0.25	0.06-0.125	0.12-0.25
Mycoplasma pneumoniae	0.001	0.008-0.03	0.004

a   Erythromycin-resistant organisms will be resistant to erythromycin alone.

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Using the strategy of the present invention, it also
possible to use other antimicrobial agents in combination
with the MLS antibiotics and inhibitors described herein.
The use of antibiotic combinations is well established,
for example, see Eliopoulous & Moellering (1991;
incorporated herein by reference). Combinations are used
primarily for two reasons: to provide broad-spectrum
coverage in patients that are seriously ill and who may
be come septicemic and, less frequently, because an
identified organism is resistant to traditional doses of
single antibiotics, but which the antibiotic combination
may give the desired antimicrobial effect. Here it
should be noted that the reversal of MLS antibiotic
resistance is not discussed in (Eliopoulous & Moellering,
1991), highlighting the need for a new development in
this area.

Mechanistic considerations are not important to
practicing the invention, with the appropriate antibiotic
doses and times for administration being generally known
to those practicing in this area (e.g., defined doses at
two, three of four times per day). However, the
inventors did consider the underlying mechanisms in
choosing certain currently preferred modes of
administration in regard to MLS antibiotics and
methylation inhibitors.

The inventors reasoned that bacterial cells
generally contain 10^3-10^4 ribosomes, that the induction
of methylases reach a steady state of about 10^3-10^4
molecules in about 2-3 hours, and that induction
stabilizes the erm mRNAs half life from 2-3 minutes to 45
minutes (Denoya et al., 1986). Additionally it has been
shown that the first resistant cells appear in about
20 minutes. From the known rate of peptidyl transferase
catalysis (Devlin, 1986) and the rate of rRNA

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methylation, it is clear that inductive methylase
synthesis occurs rapidly, and it is estimated that most
of the ribosomes are methylated within one generation.

5   Following the induction of methylase synthesis,
resistance to erythromycin rapidly occurs.
Theoretically, principles suggest that to reduce or
reverse erythromycin resistance, the best treatment
regimen will be when (a) the induction of the synthesis
10   of the methylase is prevented and/or (b) when methylation
of the rRNA is prevented. Both of these can be achieved
in a number of ways, using appropriate inhibitors, e.g.,
where the concentration of the methylating agent SAM is
decreased. A preferred treatment will involve the pre-
15   administration of an inhibitor for a given time period
sufficient to decrease both protein synthesis and the
concentration of SAM, then followed by the administration
of the antimicrobial alone or in combination with more of
the same or further inhibitor(s).
20

EXAMPLE II
Evaluation of Antibiotic Susceptibility

Disk Susceptibility Test
25

           To more precisely define appropriate doses of
inhibitors, or second agents, for use with MLS
antibiotics, the inventors contemplate using a disk
susceptibility test. Disk susceptibility testing is
30   widely used in clinical laboratories (Acar & Goldstein,
1991; Shungu, 1991). The principle of these methods
involves the use of a constant concentration of an
antibiotic within a reservoir in the agar that is used to
culture the organisms of interest. The susceptibility of
35   the organism to the antibiotic is indicated by a clear
zone of inhibition around the reservoir. The diameter of

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the zone of inhibition is proportional to the
susceptibility of the organisms in question. The
diameter of the clear zone of bacteria in the presence of
antibiotics is known at the minimum inhibitory

5   concentration (MIC), therefore bacterial strains
resistant to an antibiotic can be identified quite easily
from the diameter of the clear zone; resistant bacteria
having a small clear zone diameter. The diffusion
technique developed by Bauer et al. (1966) is the

10   technique also recommended by the FDA and has been the
technique most widely used in the clinical laboratory.
This method is endorsed by the WHO and the National
Committee for Clinical Laboratory Standards (NCCLS, see
NCCLS Document, 1993).

15

The effect of more than one antibiotic, or other
agent, on a bacterium is also easily examined with agar
disk susceptibility testing. In one such example for use
with the present invention, one would use two disks, one

20   each impregnated with an MLS antibiotic, and the other
with an inhibitor of RNA methylation. An increased clear
zone will be observed where the antibiotic and second
agent interact to exert their combined effect.

25   **Checkerboard Method**

The checkerboard (or chessboard) method may also be
used to assess antimicrobial combinations. The term
"checkerboard" refers to the pattern (of tubes or

30   microtiter wells) formed by multiple dilutions of the two
antimicrobials being tested in concentrations equal to,
above, and below their minimal inhibitory concentrations
(MICs). Also included is a row (or column) of tubes (or
microtiter wells) without any antimicrobial for each

35   drug. Thus, (compound A), being diluted along the
x-axis, and (compound B), being diluted on the y-axis,

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results in squares on a checkerboard containing a unique combination of the two compounds being tested.

5   This technique may be performed with liquid or semisolid (agar) media, with microtiter trays rather than racks or test tubes, using other than 2-fold dilutions, using more than two drugs, and with organisms other than bacteria. In the agar dilution method, the principle of the technique is the same, but it is adapted for use in

10   an agar dilution system, which is advantageous when a large number of strains are to be tested against a limited number of antibiotic combinations. Microtiter trays are often preferred because they are less cumbersome and require smaller quantities of

15   antimicrobials and broth.

After conducting a checkerboard analysis, an isobologram is constructed as follows: For each concentration along the x-axis (plotted as the x-

20   coordinate), the lowest concentration of the compound diluted along the y-axis that inhibits growth in the column of tubes is taken as the y-coordinate of this plot. An isobologram is constructed by connecting the series of coordinate points generated for each drug

25   combination.

In interpreting the results, the critical question is usually whether an apparently synergistic combination is significantly below the additive line of the

30   isobologram. Because the margin of error in these studies is ± 1 dilution, a combination should be at least 2 dilutions below the additive line of the isobologram to be significantly synergistic, or at least 2 dilutions above the additive line to be significantly antagonistic.

35

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Killing Curves

Killing curves (time-kill curves, time-kill plots)
may also be used. In contrast to the checkerboard, which
5   typically provides only inhibitory data, the killing
curve technique measures the microbicidal activity of the
combination being tested. For this reason, it is
advantageous for clinical situations in which
bactericidal therapy is desirable. The other major
10   advantage of killing curves over the checkerboard is that
they provide a dynamic picture of antimicrobial action
and interaction over time (based on serial colony
counts), as opposed to the checkerboard, which is usually
examined only once (after 16 to 24 hours or incubation).
15

When the colony counts in a killing curve study have
been determined, the result are plotted and interpreted
by the effect of the combination in comparison with the
most active single drug alone. Results from
20   bacteriostatic tests are interpreted as follows: Drug
combinations are considered synergistic if inhibition
occurs with concentrations at or below the curve
described by the original MIC of each single drug and ¼
the MIC for both drugs. When an organism is sensitive to
25   the combination at concentrations ≥2x the original MIC of
each single agent, the antibacterials are designated
"antagonistic." The points that fall between synergy and
antagonism are defined as additive or "indifferent."

Serum Bactericidal Testing
30

The serum bactericidal titer may be determined and
used as an estimate of the activity of antimicrobial
combinations in patients receiving them. The advantage
35   of this approach is that it measures the activity of
antimicrobial concentrations that are achievable *in vivo*.

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Kinetic Spectrophotometric Methods

Spectrophotometric methods provide both a more
5   kinetic view of antimicrobial interaction and a more
quantitative analysis of the dose-response relationship.
In these methods, one can plot growth curves for multiple
cultures simultaneously and examine the effects of
different antimicrobials and antimicrobial combinations
10 on the growth-rate constants of those cultures. The
expected 50% inhibitory concentration (ED_{50}) of one
compound can be compared to that in combination with a
steadily increasing series of concentrations of a second
compound.

15

Paper Strip Diffusion

In the paper strip diffusion method, using
cellophane or membrane filter transfer, filter paper
20 strips are soaked in antimicrobial solution and placed at
right angles to one another on an agar plate. After
incubation, the filter paper strips are removed, leaving
behind the compounds that have diffused into the agar
medium. Following removal of the filter paper strips, a
25 transferable material (cellophane or a filter membrane)
that will permit diffusion of the two compounds now
contained on the agar plate is placed on the agar surface
and inoculated with a suspension of the organism to be
tested. After incubation, the transferable material is
30 removed from the agar plate containing the compounds and
transferred to another agar plate without antimicrobials.
Following an additional incubation, the growth pattern is
examined.

35   Additive or indifferent combinations show no
enhancement of their zones of inhibition at the former

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junction of the filter papers. Synergistic combinations
show enhanced inhibition and may even demonstrate
activity at this point, even when the organism is
resistant to both compounds separately.

5

The methods of sections 2-6 above are even more
fully described in Lorian (1991, pages 432-449).

Animal Tests for Activity and Toxicity

10

In vitro tests are useful as the starting point in
the development of new drugs and combinations for use in
treatment. However, no such drug or combination of
agents should then be administered to a human patient
15   solely on the basis of *in vitro* activity. This invention
provides inhibitory compounds for use in combination with
antimicrobials, which compounds have been selected on the
basis of reported functional characteristics. As no such
inhibitory compound is selected randomly, it is expected
20   that the correlation between *in vitro* and *in vivo* data
will be high.

However, the correlation between the strength of the
in vitro and *in vivo* activities of a compound may not be
25   linear. Therefore, certain compounds with high *in vitro*
activity may not prove to be the most advantageous for *in
vivo* use. This is not a drawback to the present
invention, but simply reflects a checkpoint that is known
to exist in all antimicrobial testing. Lorian (1991)
30   pointed out that *in vitro* and *in vivo* tests differ in
their general characteristics and specific variables.
Consequently, discrepancies are likely to occur.
However, they may be understood and interpreted correctly
if the limitations of the test systems are understood.

35

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As an example, Lorian (1991) discussed a case where discrepancies between *in vitro* and *in vivo* results could be resolved by using a concentration of drug *in vivo* that resulted in plasma levels of the antibiotic similar to
5   those used in the *in vitro* evaluation. Thus, while *in vitro* evaluations such as those used by the inventors serve as an excellent indicator of *in vivo* antimicrobial potential, it is well understood in the art that optimizing an *in vivo* dose often takes more than a direct
10   application of the *in vitro* data. Extrapolation of data to account for variable parameters such as blood volume, tissue accessibility and plasma half life is routinely practiced in the art.

15   Part of the practice of developing the *in vitro* data for *in vivo* clinical use involves studies in animal models. Lorian (1991) indicated that animal studies with antibiotics are important for suggesting appropriate indications, determining potential toxicity problems and
20   providing insight into the pharmacokinetic properties of new agents in relation to those of known agents.

In evaluating any new antimicrobial or combination thereof, it is well known that the doses necessary to
25   control the disease do not produce toxic reactions worse than those of the disease. Thus, the relationship between toxicity and efficacy must be ascertained. This relationship is known as the therapeutic index. The larger this index, the less likely it is that the drug or
30   combination will cause toxic effects when used clinically. An index of 10 is often regarded as a safe minimum for an antibacterial, and this guideline can be used in the present invention in combination with the data given herein. In experimental studies, the index is
35   usually calculated as the ratio of the dose that kills

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50% of the animals (LD_{50}) to the dose that protects 50% of animals (PD_{50}).

In analyzing the amount of drug to be administered
5   during animal tests, the maximum tolerated dose of a
substance should be determined (using the most suitable
route of administration). Before use in the treatment of
infected animals, the maximum tolerated dose is
determined by administering single injections of the
10   substance to groups of animals by the oral, subcutaneous,
or intraperitoneal routes. The animals are then observed
for survival for periods of 24 hours to 7 days. Such
acute toxicity studies establish for each route the 100%
toxic dose (LD_{100}), the 50% lethal does (LD_{50}), and the
15   maximum tolerated dose (LD_0) at which all animals
survive. Experience has allowed the formulation of the
rule that approximately 1/5 of the maximum tolerated dose
of a substance will be tolerated when treatments are
given once daily for 5 days or longer. For 1 to 3 days
20   of treatment, 1/2 the maximum dose can usually be given.

In addition to obtaining initial information on
toxicity, some information on absorbability may also be
obtained. For example, if a substance is tolerated at
25   1000 mg/kg when given orally, but toxic when given at a
dose of 50 mg/kg intraperitoneally or intravenously, the
lack of toxicity by the oral route probably reflects poor
oral absorption.

30   Interspecies variation in response to drugs is known
to occur and this possibility must be considered when
predicting the effect of the drug in man. If several
species of animals are used for the toxicity studies, and
the same toxic effects occur in all the species, then it
35   is likely that these effects will also occur in man.
Conversely, if no toxic effects are observed in any

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species of animal, except with unrealistic doses, then none are likely to occur with therapeutic doses used in man. The animals most commonly used in toxicity studies are the mouse, rat, rabbit, dog, and monkey. By

5   utilizing animal models known in the art, as exemplified by those described herein, the combinations of the present invention can readily be developed for use in the clinical environment.

10   The inhibitors and antibiotic/inhibitor combinations identified by the inventors should therefore be evaluated *in vivo* in small animals in a fashion similar to that described by Lorian for new antibiotics (Lorian, 1991, pp. 746-786, incorporated herein by reference). It is

15   important to note that since this invention generally concerns new and surprising combinations of known compounds, the testing needed will likely be less vigorous than that needed with a completely new drug. The toxicity data known for any individual drugs may be

20   used as starting guidelines, and combined doses below such levels used in studies to confirm the advantages of the proposed combinations.

As described by Lorian (1991), animal models most

25   frequently used in the evaluation of antibacterials may be categorized as basic screening, *ex vivo*, monoparametric, and discriminative. The *ex vivo* and monoparametric models are used to measure specific variables (e.g., dosage schedule, serum binding, or

30   penetration into extravascular spaces). The discriminative systems are employed to differentiate the new agents from related or unrelated active agents. For preliminary evaluation of new agents and combinations, as in this invention, the basic screening system is usually

35   employed.

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Screening models involve simple one-step infections, simple techniques and schedules of treatment, short-duration experiments, reproducible courses of infection, simple evaluation (all-or-nothing models), economy of

5   test drugs, and low costs. These requirements are best met by the mouse protection test, which is the most widely used *in vivo* screening model in antibacterial research. The mouse protection test is suitable for determining efficacy and toxicity of new antibacterials

10   and it can indicate whether a drug or new combination is likely to be active orally or parenterally. The manner of conducting such tests will be well known to those of skill in the art in light of the present disclosure and the guidelines set forth below.

15

In a typical mouse protection test, the primary host used is the Swiss albino mouse. This is because there is a good correlation between clinical response to an antimicrobial and activity in the mouse; large numbers

20   are easily obtained at relatively small cost per unit; and because an outbred strain of mice provides a heterogeneous population, which allows for immunological and other host factor variations.

25   In developing an experimental mouse model for *in vivo* testing, it is desirable to use human pathogens whenever possible. It is also desirable to infect with strains of microorganisms that are sufficiently virulent so that conditioning procedures to lower the host's

30   resistance are unnecessary. When reproducible infections cannot be achieved by inoculation of the organisms alone, adjuvant stressing measures can be used to lower the host's resistance. In the case of infections with *Staphylococcus aureus*, a commonly employed measure is to

35   suspend the organisms in 3 to 10% hog gastric mucin and

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to administer 0.5 ml amounts by the intraperitoneal
route.

    In preparing a new agent for testing, the stability,
5 degree of solubility and/or the means by which a uniform
suspension may be prepared should be considered. If the
antibacterial is insoluble and is to be administered by
the oral route, it will generally be prepared in a
solution containing 1% carboxymethylcellulose. If an
10 insoluble agent or combination is to be administered by
the subcutaneous or intraperitoneal route, a suspension
in water is prepared by sonication or homogenization. If
these methods fail to produce a uniform suspension, the
material is dissolved in DMSO or ethanol. The resulting
15 solution is then diluted to a final concentration of less
than 10% DMSO or ethanol, since higher concentrations of
these solvents are lethal to mice. For treatment,
antibacterials should be freshly prepared each day.

20   In order to obtain reproducible infections, it is
necessary to determine the degree of virulence of each
strain to be studied. The lowest dilution of the
organisms at which all the animals die is defined as the
minimum lethal dose (MLD). Alternatively, in place of
25 MLD one can use multiples of the challenge dose of
organisms that kills 50% of the animals (LD_{50}). After
the animals are infected and treated, they are observed
over a fixed period and the number of survivors is
recorded. The 50% protective dose (PD_{50}) is calculated
30 in mg/kg. The 50% protective dose is the same as the 50%
curative dose (CD_{50}) or 50% effective dose (ED_{50}). By
altering the treatment route or schedule, differences in
activity can be demonstrated. In addition, the relative
efficacy of oral and subcutaneous administration of a
35 substance can be compared.

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The mouse protection test has been studied in great detail, and evaluation of antibacterials using this method has produced a number of clinically effective agents (Grunberg & Cleeland, 1977). Various studies have
5   shown that using the mouse protection test the PD_{50} values generally fall into the same ranges as the clinical doses measured in mg/kg. However, this is not an indication that the doses in the mouse models should be exactly the doses employed in the clinical management
10   of infections. Certainly humans are not treated on the basis of achieving only 50% survival. In spite of this, there is a reasonable overall correlation between the results obtained in the mouse model and clinical effectiveness.
15

Many agents that are not active against systemic infections are active against local infections when applied at the site of infection. Different techniques for producing local infections have been described, with
20   the basic principle being the induction of a localized infection that is treated by application of the desired agent to the infected site. Antibiotics and combinations known to be inactive when given orally or subcutaneously have been shown to be active when infiltrated into the
25   site of local infection, as exemplified by the activity of mycin against gram-positive organisms and two gram negative strains (Grunberg et al., 1967).

In the mouse protection tests described above, the
30   infections are primarily of short duration, unnatural and overwhelming or self-limiting. A number of specific infections in a variety of animal hosts have been utilized to mimic human infections more precisely and will be known to those of skill in the art. Examples
35   that appear to be reasonably faithful to the human infection are pyelonephritis in the rat and meningitis,

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endocarditis and osteomyelitis in the rabbit. In a
number of other experimental infections, the targeting of
infection to specific organs, while not as close in
mimicking the human situation (e.g., meningitis and
5   pneumonia in mice), provide useful models for predicting
the selectivity of new agents and combinations.

    The following is a description of certain exemplary
discriminative animal models that may be of use in
10   connection with the present invention. For most of the
discriminative animal model systems it appears that the
agents shown to be very effective experimentally are
generally useful clinically (Ernst & Sande, 1981).

15   A simple model for the formation of intraperitoneal
abscesses in mice is available that offers opportunities
for studying various aspects of staphylococcal host-
parasite interactions occurring in localized lesions. As
an example of the formation of abscesses by anaerobes, a
20   model of subcutaneous abscesses in mice caused by
Bacteroides fragilis has also been described.

    The thigh lesion model provides a nonlethal
experimental infection to evaluate the effectiveness of
25   an antimicrobial combination and allows the measurement
of drug-pathogen interaction and drug pharmacokinetics in
the infected host. If the animals are made neutropenic,
then the thigh model becomes an excellent system for
measuring the drug-microorganism interaction with most of
30   the host defense system eliminated (Vogelman et al.,
1988).

    Regarding respiratory tract infections, such as
pneumonia, various models are available. For example, in
35   the mouse model of pneumonia, disease is induced in mice
by intranasal instillation of 3 drops of an undiluted

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overnight culture of *Streptococcus pneumoniae* 6301 in
trypticase soy broth containing 5% goat serum (Beskid
et al., 1981). A useful model for the production of
pneumococcal pneumonia in rats has been described by
5   Ansfield *et al.* (1977). For the detection of
antitubercular drugs, a number of mice systems have been
described. An infection in guinea pigs useful for
testing new antitubercular agents is also available. In
this model. *M. tuberculosis* H37Rv, whether inoculated by
10   the subcutaneous or intramuscular routes, produces a
progressive, predictable disease.

Models for experimental acute urinary tract
infections are commonly used to evaluate antibacterials.
15   In mice, these infections produce either hematogenous or
ascending infections, depending on whether the inoculum
is administered intravenously or intravesically, with or
without the addition of a foreign body. In the mouse
pyelonephritis model, the infected animals, in most
20   instances, either die or heal spontaneously.
Consequently, treatment has to be started shortly after
infection in order to alter the course of disease. For
chronic pyelonephritis, agents are sought that will
control the disease after the pathological damage has
25   occurred. An infection in rats which stimulates chronic
E. coli nephritis has been described in Ryan (1976).

A number of systems for studying the effect of
antibacterials in experimentally induced meningitis have
30   been reported. A simple lethal mouse model to produce
pneumococcal meningitis is available (Tsai *et al.*, 1975).
For induction of meningitis, Swiss albino mice were
subjected to strains of *S. pneumoniae* virulent for mice
by the intraperitoneal route. A model for the induction
35   of *H. influenzae* b meningitis in infant rats, which
appears to be both simple and reproducible, has also been

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described. An excellent rabbit model used to study the
activity of antibacterials in the therapy of experimental
meningitis has been described using New Zealand white
rabbits (Dacey & Sande, 1974; Scheld et al., 1978).

5

The method described by Garrison & Freedman (1970)
for inducing experimental endocarditis in rabbits has
been well studied and shown to be a reliable model for
evaluation of the effectiveness of antibacterials. The

10   rabbit model described by Norden (Norden, 1970) is a
simple and reproducible system for the study of
osteomyelitis. A rat model for simulating intraabdominal
sepsis, either with known organisms or with mixed fecal
flora cultures, has been developed by Onderdonk et al.

15   (1974).

A simple model for keratitis and experimental
deratitis in guinea pigs is available (Davis et al.,
1977), as is a simple method to obtain reproducible

20   intraocular infections in rabbits (Maylath & Leopold,
1955).

Models are even available for sexually transmitted
diseases. Corbeil et al. (1979) have described a mouse

25   model for disseminated gonococcal infections, using a
gonococcal strain (N24) isolated from a human genital
tract. The most successful model for studying gonococcal
urethritis is produced by the inoculation of chimpanzees
with human urethral exudate (Arka & Balows, 1986).

30   Johnson et al. (1982) have described a method for
experimentally inducing syphilis in rabbits.

Models of fungal infections are also available,
e.g., for infection with fungi other than dermatophytes,

35   intravenous injection of clinical specimens or culture
isolates is used for the conversion of dimorphic fungi to

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the tissue phase. Overwhelming numbers of organisms are
usually used, since such infections develop slowly and
frequently are not fatal. An example of a non-fatal
subcutaneous fungal infection model is that achieved by
5 accomplishing a local infection with *C. albicans* in
cortisone-preconditioned animals. Such models may be
employed in the detection of topical antifungal agents
and combinations.

10 Preferably, in the above models, animals infected
with resistant microorganisms would be treated in at
least two ways before progressing to clinical trials. In
the first procedure, the infected animals will be treated
with the inhibitor(s) for a predetermined time before the
15 antibiotic(s) treatment is started. In a second
procedure, the infected animals are treated with the
antibiotic(s)/inhibitor(s) combination at the same time.
In yet another procedure, the first regimen could be
modified where the infected animals would be treated pre-
20 treated with the inhibitor(s) alone, then followed by
treatment with the combination of the
antibiotic(s)/inhibitor(s).

EXAMPLE III
25 <u>**Methylation Inhibitors Increase Antibiotic Susceptibility**</u>

To show that compounds capable of directly or
indirectly inhibiting methylation were able to increase
the susceptibility of bacteria to MLS antibiotics, the
30 inventors first used methods similar to the disk
susceptibility test described in Example II.

In both of the following procedures, the protocol
involved obtaining *S. aureus* lawn cultures in agar Petri-
35 dishes. Freeze-dried *S. aureus* cultures from the ATCC
[Strain 27660 (inducible resistant strain); strain 6538

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(susceptible strain)] were reconstituted in 5 ml sterile
broth media.   Either Trypticase Soy Broth (TSB, Casein
peptone 17g, soya peptone 3g, dipotassium phosphate 2.5g,
NaCl 5g and dextrose 2,5g, Lot X1582, Adams Scientific
5   Inc., West Warwick, RI) or Brain Heart Infusion Broth
(BHIB, Casein peptone 15g, meat peptone/BHI solids 12g,
dipotassium phosphate, Dextrose 2.0g, yeast extract 5.0g
and NaCl 5.5g, Lot DM1082, Scott Laboratories Inc., West
Warwick, RI) were used, at concentrations of 30 and 42g/l
10   respectively.

      The inoculated broths were incubated for 24h at
37°C, when they were streaked out on appropriate agar
plates to assess purity of culture.   Either of 40g/l BBL
15   Trypticase Soy Agar (TSA, Pancreatic digest of casein
15g, papaeic digest of soybean meal 5g, NaCl, 5.0g and
agar 15g, Lot K6DCXY, Becton and Dickenson, Cockeysville,
MD) or Brain Heart Infusion Agar (15g/l Agar, Scot
Scientific Inc. West Warwick, RI, Lot C-0281 and 42g/l
20   BHIB) were used.

      In the first procedure, two BHIA agar plates (Falcon
Petri-dish 50 mM, Becton Dickenson, Lincoln Park, NY)
were prepared, with an inhibitor mixed into one of the
25   plates at the appropriate concentration.   Both plates
were inoculated with, e.g., a *Staphylococci* species
resistant to erythromycin, using a sterile, aseptic
wooden cotton swap containing about 0.2 ml of a 10^6
organism/ml solution.   Two disks, each impregnated with
30   erythromycin (15 mg/disc; Remel, Lenexa, Kansas), are
placed on each agar plate.   The plates were incubated for
17-24 hours then the clear zones were analyzed.

      Although BHIA contains about 3% protein in contrast
35   to the 2% protein in TSA, it is suspected that TSA
contains higher concentrations of methionine and/or

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growth factors that increase the robustness of *S. aureus*
to erythromycin.

5   FIG. 1 shows the results from one such study in
which erythromycin susceptibility for *Streptococcus
aureus* 6538 (erythromycin susceptible) was examined in
the presence of erythromycin in combination with
ethionine, cycloleucine and trimethoprim. FIG. 2 shows
similar results but using the erythromycin resistant
10   strain *S. aureus* 27660. Ethionine, cycloleucine and
trimethoprim all result in an increase in the size of the
clear zone.

       In a second evaluation procedure, 100mM Petri-dish
15   agar plates (American Precision Plastic, North Geln, Co.)
were prepared, as described in the first procedure, but
this time with no inhibitor pre-mixed into the agar.
Five discs containing 310 μg ethionine/disc containing
disc were placed on the *S. aureus* (resistant) incubated
20   BHIA agar plate at zero time. At times 0, 1, 2, 3, and 5
hours, each disc was then removed and replaced with an
erythromycin disc at the identical position that the
inhibitor disc had previously occupied. Erythromycin
controls were included at each point.
25
       Increases in clear zones were observed after 18
hours incubation with inhibitor (ethionine)/erythromycin
combination relative to erythromycin alone (FIG. 3).
From these results it appears that pre-incubation with
30   inhibitor for increased time periods increases the
sensitivity of the resistant organisms to erythromycin.
It is also contemplated that a broth susceptibility test
will be suitable for analysing the effects of inhibitors.

35   Using the first procedure described above, various
other inhibitors were also tested. At concentrations of

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between 18 mM (for methotrexate) and 410 mM (for
nicotinamide), all of the agents except SAH tested proved
to increase the size of the clear zone (Table 8). The
results are expressed in terms of relative effectiveness.

TABLE 8
ERYTHROMYCIN SUSCEPTIBILITY FOR *S. AUREUS* 27660

Inhibitor	Concentration mM	Clear Zone mM	% Relative effectiveness[3]
Control	0	10^2	100
Methylthioadenosine	42^1	12.5	183
Nicotinamide	410	12.5	183
Na$^+$ Sulfadiazine	56	12.5	183
3-Deazaneplanocin A	31	12	167
2-Deoxyadenosine	183	14	233
DFMO	46	14.5	250
Ara-A	39^1	12.8	193
D,L-Buthionine-(S,R)-Sulfoximine	90	12	167
SIBA	33^1	12.8	193
Tubercidin	34^1	12.8	193
Methotrexate	18	14	233
S-Adenosylhomocysteine	35^1	10	100
2-Chlorodeoxyadenosine	35^1	12	167

[1] Contained 16% Dimethylformamide (DMF)
[2] Controls with and without DMF identical
[3] $[D_{mod}\cdot D_{disc}]/[D_{Cont}\cdot D_{Disc}] \times 100$

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As shown in Table 8, with the great majority of the inhibitors, the bacterial lawn was observed to be substantially inhibited in comparison to the controls with no inhibitor. It is more than likely that SAH is
5   not taken up by *S. aureus*. Walker & Duerre (1975) showed with studies in dogs that ^{3}H-labeled SAH is not taken up and excreted unchanged in urine. There exists a high likelihood that a similar effect occurred in the present study with SAH.
10

Importantly, the inhibitors shown in Table 8 are generally representative of the different groups of inhibitors identified by the inventors, i.e., compounds capable of inhibiting RNA methyltransferases; SAH
15   hydrolase; SAM synthetase (via inhibiting glutathione synthetase); dihydrofolate reductase (DHFR); and also, agents that inhibit polyamine synthesis.

It should be noted here that the inventors currently
20   contemplate that the use of other analytical methods will allow the above enhancement data to be refined. This is because the disc testing protocol, while clearly very useful, is not considered by the inventors to be the optimal protocol for a quantitative type of study. As
25   such, a broth culture procedure, where the organism is grown in the presence of both erythromycin and the inhibitor in liquid medium, is proposed. The growth of bacteria is then assessed photometrically (Example II). This procedure is contemplated to allow a better
30   evaluation of the potency of the inhibitors.

As a reference point, it is important to note that erythromycin, as an example of an MLS antibiotic, is used clinically at an oral dose of 2000 mg/day (Reese & Betts,
35   1993). *In vitro*, in an agar disc susceptibility test, a MIC_{90} (minimum inhibitory concentration for 90%

- 87 -

inhibition) of about 4 μg/ml for *S. aureus* is typically
determined, while the MIC in broth culture for the same
organism is typically between 0.12 and 0.5 μg/ml
(Weideman & Atkinson, 1991).

5

EXAMPLE IV

Inhibitors of Transmethylases

SAH and SAH Analogues

10

SAH hydrolase (SAHH) occurs in many procaryotic
organisms (Shimizu *et al.*, 1984). It is believed that
SAHH plays an important role in many cells in removing
SAH, a product of transmethylation reactions involving
15 SAM (Thong *et al.*, 1985). SAH is an inhibitor of most
SAM-dependent transmethylase reactions, consequently
inhibition of SAHH gives rise to higher concentrations of
SAH which reduces the activity of the transmethylases.

20   Yebra *et al.* (1991) showed that SAH (100 μM) and
sinefungin (50 μM) inhibit RNA methyltransferase from
Streptomyces antibioticus by 97 and 96% respectively.
From these results, the inventors estimated that SAH
dosage ranges similar to that of sinefungin could be used
25 clinically. Due to the membrane transport
characteristics of SAH it is anticipated that the most
advantageous method of SAH delivery will be as a liposome
or nano particle (Couvreur *et al.*, 1991; Example XIII).
Since the net effect of SAHH inhibition is to raise the
30 SAH concentration which causes the inhibition of
transmethylation reactions, it is expected that SAH will
be used alone or in combination with other methylation
inhibitors. Doses useful in therapy with antibiotics,
will be from about 1 mg/kg/day to about 100 mg/kg body
35 weight/day, and preferably, from about 5 to about 50
mg/kg body weight/day.

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Coward et al. (1974) reported that 2-fluoro-
S-adenosylhomocysteine (2-FSAH) is an effective inhibitor
of SAHH. Other structural analogues of SAH with
modification in the amino acid, base or sugar portion of
5   the molecule have been evaluated (Pugh & Bochardt, 1982)
in vitro for their abilities to inhibit the SAM-dependent
transmethylations catalyzed by vaccinia virion
mRNA(guanine-7)methyltransferase and
mRNA(nucleoside-2'-)methyltransferase. S-
10   Tubercidinylhomocysteine (STH) was found to be a
particularly potent inhibitor of
(nucleoside-2')methyltransferase.

From the candidate inhibitors of vaccinia virion
15   mRNA(guanine-7)methyltransferase evaluated at 100 μM,
Pugh & Bochardt (1982) showed that S-adenosyl-L-
homocysteine sulfoxide (47% inhibition), S-adenosyl-L-
homocysteine sulfone (40%), S-(N^6-methyladenosyl)-L-
homocysteine (85%), S-(3-deazaadenosyl)-L-homocysteine
20   (84%), and S-aristeromycinyl-L-homocysteine (66%),
sinefungin (100%) and A9145c (100%) were the most potent
analogs.

Gillet et al. (1979) also described a number of SAH
25   analogues mainly modified in the amino acid portion of
the molecule, that are all effective competitive
inhibitors of protein methyltransferase II from human
erythrocytes. From these analogues, the inventors
propose that 5'-S-(3-carboxyl-4-
30   nitrophenyl)thioadenosine, S-adenosyl-L-homocysteine
sulfoxide and 5'-S-(methyl)-5'-S-(butyl)thioadenosine
would be particularly useful in connection with this
invention.

35   The following doses are proposed for use in killing
bacteria, and other microorganisms, in combination with

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antimicrobial agents: between about 1 to about 200, and
preferably, between about 10 to about 100 mg/kg body
weight/day for 2-FSAH and S-aristeromycinyl-L-
homocysteine (STH); between about 1 to about 200, and
5   preferably, between about 10 to about 100 mg/kg body
weight/day for S-Adenosyl-L-homocysteine sulfoxide and
S-Adenosyl-L-homocysteine sulfone; and between about 1 to
about 200, and preferably, between about 5 to about 75
mg/kg body weight/day for S-(N^6-methyladenosyl)-L-
10   homocysteine and S-(3-deazaadenosyl)-L-homocysteine.

Homocysteine

Homocysteine is mainly methylated by two different
15   enzymes in the last step of the biosynthetic pathway in
bacteria to form methionine, which is essential for the
synthesis of SAM. The two enzymes are homocysteine
methylase [EC 2.1.1.14; using N^5-methyl
tetrahydropteroylglutamate (N>2) as methyl donor] and
20   homocysteine methylase [EC 2.1.1.13; using vitamin B12 as
methyl donor]. E. coli, as an example, contains both
enzymes, whereas most organisms possess only one enzyme
(Cohen & Saint-Girons, 1987). The inhibition of any of
the biosynthetic reactions involved in the synthesis of
25   homocysteine, is predicted by the inventors to inhibit
transmethylation reactions since it increases the SAH/SAM
ratio.

S-N^6-methyladenosylhomocysteine
30

S-N^6-methyladenosylhomocysteine is nearly as potent
an RNA methyltransferase inhibitor as SAH (Hoffman,
1978). Administration of N^6-methyladenosine has been
proposed as a general method for blocking in vivo RNA
35   methylation in studies to determine the role of
methylation in RNA processing and translational function.

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S-N^6-methyladenosylhomocysteine may be synthesized *in vitro* from N^6-methyladenosine and homocysteine in a reaction catalyzed by S-adenosylhomocysteine hydrolase.

5   S-N^6-methyladenosylhomocysteine is commercially available from Sigma Chemical Company and may be used, in combination with an antimicrobial agent at doses of between about 1 to about 100 mg and preferably, between about 5 to about 50 mg/kg body weight/day.

10

Analogues of S-Aristeromycinyl-L-Homocysteine

       A series of base- and amino acid modified analogues of S-aristeromycinyl-L-homocysteine, a carbocyclic
15   nucleoside, were synthesized and evaluated as inhibitors of SAM-dependent methyltransferases (Houston *et al.*, 1985a). Houston *et al.* (1985a) observed that from the evaluated compounds S-aristeromycinyl-D-homocysteine and 3-deazaaristeromycinyl-D-homocysteine were particularly
20   effective methylation inhibitors with K$_i$ of 10.4 and 20.5 μM, respectively, for phenylethanolamine N-methyltransferase.

       These carbocyclic analogues are believed to be
25   improved inhibitors *in vivo* as they will be more stable to metabolism than their ribosyl analogues (Houston *et al.*, 1985a). Doses of between about 1 to about 100, and preferably, between about 5 to about 50 mg/kg body weight/day are proposed for these analogues in
30   combination with antimicrobial agents such as MLS antibiotics.

       Polyinosinate

35   Liau *et al.* (1973) have shown that polyinosinate inhibits transfer and ribosomal RNA (tRNA and rRNA)

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methylases. The mechanism of inhibition has been
attributed to specific interactions between the single-
stranded polyinosinate and the methylating enzymes.
Methylation of individual bases were differentially
5   affected by poly(I): inhibition of adenine methylases
occurred to the greatest extent and guanine methylases to
the least. Local administration of 12 μg/mouse of
poly(I) every 12 hours for 15 consecutive doses has been
reported to inhibit multiplication of *Mycobacterium*
10   *leprae* in the footpad of mice (Levy & Merrigan, 1977).

Polyinosinate with molecular mass ranging from 5000-
200,000 is preferred for use in the present invention.
From the data of Liau *et al.* (1973) and Levy & Merrigan
15   (1977), the inventors contemplate that concentrations of
poly(I) useful in therapy with agents such as MLS
antibiotics, will be from about 0.1 mg/kg/day to about 30
mg/kg/day, and preferably, from about 0.1 to about 10
mg/kg/day. Poly(I) has been proposed for use in cancer
20   chemotherapy and is thus considered suitable for human
administration. Polyinosinate is commercially available
from P-L Biochemicals (Milwaukee, Wis.).

Poly(I) was also found to be particularly effective
25   in combination with adenine and adenosine (Liau *et al.*,
1973). Extending the above schemes, it is thus also
contemplated that adenine or adenosine could be combined
with poly(I), with the adenine or adenosine being in a
dose of from about 1 to about 700 mg/kg/day, and
30   preferably, of from about 1 to about 500 mg/kg body
weight/day.

S-Tubercidinylhomocysteine (STH)

35   S-Tubercidinylhomocysteine (STH) has a stable
ribose-purine bond and retains the inhibitory potency of

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SAH on tRNA methylases. The efficacy of STH parallels
that of SAH. The synthesis of STH in particular is
described in Coward *et al.* (1974) and its structure is
depicted below:

5

10

15

$$X = NH; \; Y = N; \; Z = C \; (SFH)$$
$$X = CH; \; Y = CH; \; Z = N \; (STH)$$
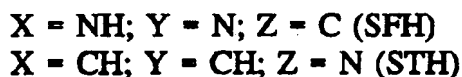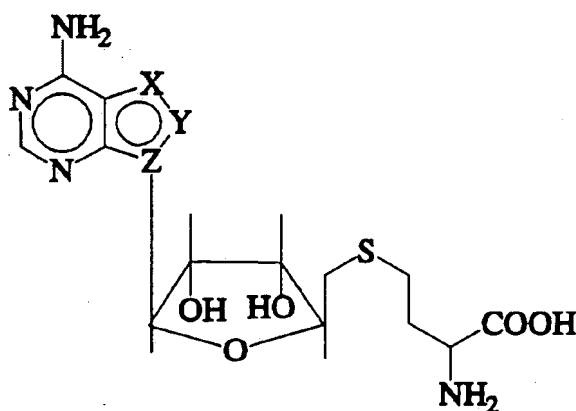
20   STH has been used to study the methylation of mRNA
in vivo. Inhibition of cytoplasmic methylation of
poly(A)-containing mRNA poly(A)-RNA methylation has been
observed using a level of inhibitor that still permitted
cell growth (Kaehler *et al.*, 1977). The use of STH as a
25 methylation inhibitor offers several advantages since it
is able to permeate cell membranes, and is not
susceptible to the nucleosidase enzymes responsible for
SAH metabolism in mammalian cells.

30   Chang & Coward (1976) also synthesized SAH analogues
in which the 5'-thioether linkage is replaced by an
oxygen or nitrogen isostere. These compounds were
designed to be resistant to enzyme-catalyzed hydrolytic
cleavage of the 5'-substituent. Although these compounds
35 showed lower inhibition of catechol O-methyltransferases,

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they are contemplated for use as inhibitors of rRNA
methylases.

5   STH is proposed for use in killing microorganisms in
combination with antimicrobials at doses of between about
1 to about 100, and preferably, between about 5 to about
50 mg/kg body weight/day; with the analogues described
above being useful in the range of about 1 to about 100,
and preferably, about 70 to about 75 mg/kg body
10   weight/day.

SAM Analogues

The synthetic approach to stable nitrogen alkyl SAM
15   analogues, in which the sulfur atom is replace by a
nitrogen atom, has been described (Minnick & Kenyon,
1988). The procedures described have been used to
prepare the methyl, ethyl, n-propyl, allyl, n-butyl,
n-pentyl, n-octyl and 6-amino-1-hexyl nitrogen analogues
20   of SAM. Testing one such nitrogen SAM analogue (in which
R = Me in the structure below) with $E. coli$ transfer RNA
(uracyl-5)-methyl transferase has revealed that this
compound functions as an inhibitor, but not as a methyl
group donor (Minnick & Kenyon, 1988). The methyl
25   analogue has been shown to have a K_i of 9 μM for the $E.$
$coli$ methionine synthase.

It is expected that these derivatives will compete
with all enzyme catalyzed reactions involving SAM. Such
30   compounds, including the nitrogen analogues described
below, are generally proposed for use in killing
microorganisms in combination with antimicrobial agents,
e.g., MLS antibiotics, at doses of between about 1 to
about 100, and preferably, between about 5 to about 50
35   mg/kg body weight/day.

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Analogues of SAM in which the sulfur atom is replaced by a nitrogen atom have the advantage that they will not be susceptible to decomposition reactions peculiar to SAM. The structures of such analogues are
5   shown:

10

15

R=Me   R=n-Bu
R=Et   R=n-Pe
R=n-Pr   R=n-Oc
R=CH₂=CHCH₂   R=H₂N(CH₂)₆

20
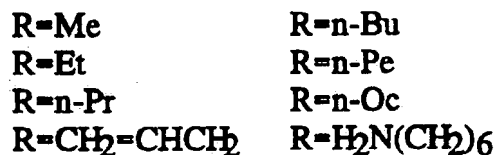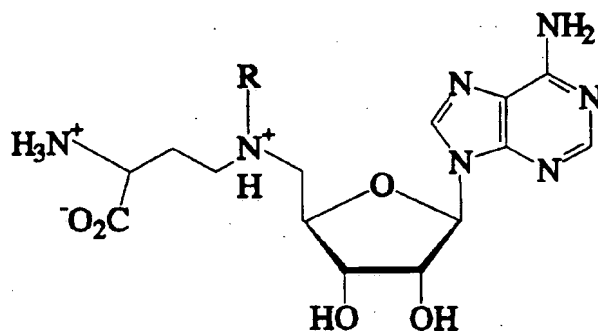
Sulphonium SAM Derivatives

Zappia et al. (1969) described the preparation, purification, analysis of a variety of sulphonium
25   derivatives of methionine. The methyl donor capacity of the inosine derivatives, and of decarboxylated S-adenosylmethionine, ranged between inactivity and effect equal to that of the biological methyl-donor SAM. In addition to SAH, these studies showed that
30   S-inosyl-L-methionine, S-adenosyl-(5')-3-methylthio-propylamine, and 5'-(methylthio)adenosine were effective inhibitors.

The structures of active SAM sulphonium analogues
35   are shown below. Such compounds, including the nitrogen analogues described below, are generally proposed for use

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in killing microorganisms in combination with, e.g., MLS
antibiotics, at doses of between about 1 to about 150,
and preferably, between about 5 to about 75 mg/kg body
weight/day.

R_1	R_2
$-NH_2$	$-CH_2CH_2CH(NH_2)COOH$
$-NH_2$	$-CH_2CH_2CH(OH)COOH$
$-OH$	$-CH_2CH_2CH(OH)COOH$
$-OH$	$-CH_2CH_2CH(NH_2)COOH$
$-NH_2$	$-CH_2CH_2CH_2NH_2$
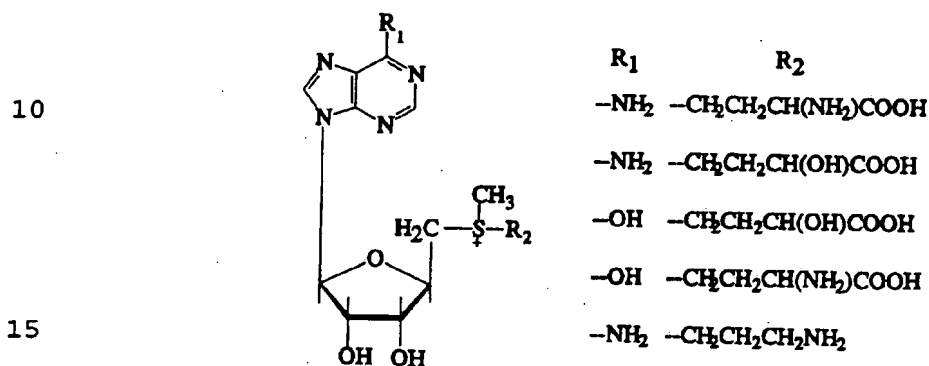
5'-deoxy-5'-S-isobutyl-adenosine (SIBA)

5'-deoxy-5'-S-isobutyl-adenosine (SIBA) is known to
be a competitive inhibitor of SAM and to strongly inhibit
methylation in whole cells (Bona et al., 1976). SIBA
inhibits the methylation of eukaryotic mRNA, especially
that of the 5' cap, and it also affects various mRNA
methylation to different extents (Jacquemont & Huppert,
1977).

SIBA also influences cell transformation by Rous
sarcoma virus. Robert-Gero (1975) reported that
adenosine homocysteine was less active as an inhibitor
than its analogues and SIBA appeared to be the most

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potent among the latter at concentrations that were not toxic to normal cells.

SIBA has a structure as shown below, and position 2 is commercially available from Sigma Chemical Company.

5

10

15
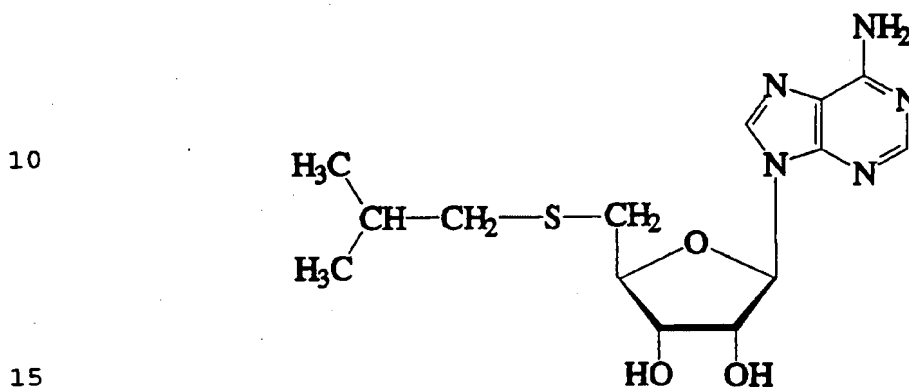
20

From established data, such as that from Jacquemont & Huppert (1977) showing that RNA methylation is inhibited ~65% by 1 mM SIBA, the inventors contemplate that therapeutically useful antibacterial concentrations of SIBA, when present with an antimicrobial agent, such as an MLS antibiotic, will be from about 1 to about 100 mg/kg body weight/day, and preferably, from about 10 to about 40 mg/kg body weight/day.

25

SIBA (0.033 M) was evaluated by the inventors and shown to enhance the relative activity of erythromycin against *S. aureus* 27660 by 193% (Table 8).

30

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Sinefungin and A9145c

        Sinefungin is an antifungal antibiotic isolated from
cultures of *Streptomyces griseolus* (Vedel & Robert-Gero,
5   1981). Sinefungin very strongly inhibits the methylation
of G in position 2 and 7, and that of A in position 1 and
6.

        Sinefungin and a related metabolite, A9145C, were
10   also found to be potent inhibitors of Newcastle disease
virion mRNA- and vaccinia virion
mRNA(guanine-7-)methyl-transferase and vaccinia virion
mRNA(nucleoside-2'-)-methyltransferase. Sinefungin and
A9145C were tested as inhibitors of these methylases and
15   both were found to be extremely active. At drug
concentration of 10 μM, where significant inhibition of
virion plaque formation was observed, no cellular
toxicity was detected. Although, at higher
concentrations of sinefungin and A9145C, significant
20   cellular toxicity was observed (Pugh *et al.*, 1978), it
has now been shown by Lemeteil *et al.* (1993) that
sinefungin could be used in a rat model against
Cryptosporidium parvum at a dosage of 10 mg/kg/day.
Additionally, Avila *et al.* (1990) reported the optimum
25   dosage using a mice model for cutaneous Leishmaniasis was
4 mg/kg/day, 50 times lower than the LD_{50}.

        Yebra *et al.* (1991) also showed that sinefungin and
derivatives strongly inhibit RNA methyltransferases from
30   *Streptomyces* (*S. antibioticus, S. incarnatus,
S. griseolus*. Additionally it was shown that sinefungin
and its analogues inhibit both RNA and DNA
methyltransferases from these organism. *S. antibioticus*
methyltransferase is inhibited at the 100 μM level by
35   A9145C (95%), cyclosinefungin (50%), SIBA 55%, ISOSIBA
(52%) and 5'-S-methylthiomethyladenosine (65%). These

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results are in agreement with Li *et al.* (1985), who
showed that sinefungin inhibits methylation of rRNA in
S.cerevisiae and leads to a decrease in stable 18S rRNA.
It is therefore expected that sinefungin and its
5   analogues will function well with antibiotics and
antimicrobials as claimed in the invention.

Sinefungin is commercially available from Sigma
Chemical Company. The structure of sinefungin, in
10   relation to that of SAH, is shown below.

15

S A H

$$HOOC-\underset{\underset{NH_2}{|}}{CH}-CH_2-CH_2-S-CH_2$$

20

OH OH
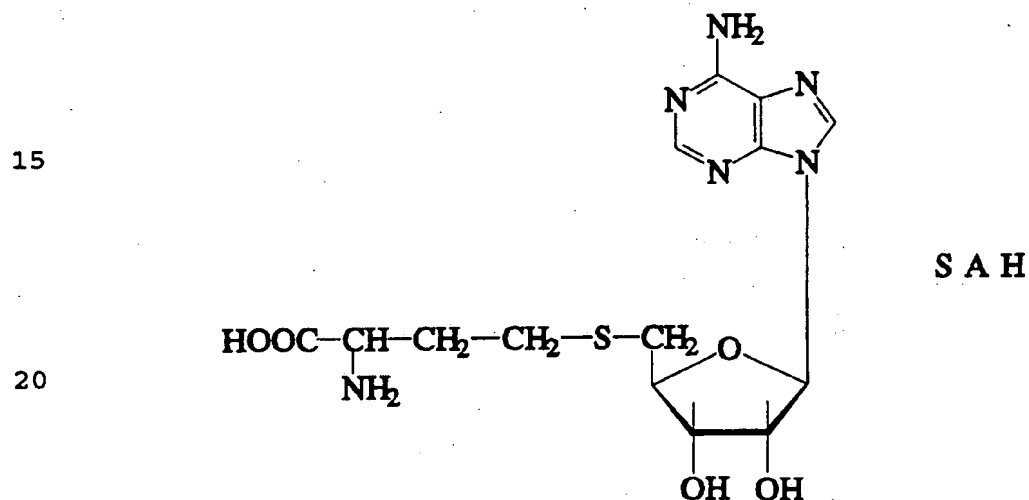
25

The synthesis of sinefungin has been reported by
Barton *et al.*, (1991) which allows modifications of the
base portion of sinefungin to be prepared. Uracil
analogues of sinefungin have also been prepared from
30   uridine and L-aspartic acid (Barton *et al.*, 1992), but
are generally believed to have decreased inhibitory
activity.

Based on the reports of Lemeteil *et al.* (1993) and
35   Avila *et al.* (1990), it is contemplated that sinefungin
concentrations of between about 0.1 and about 150 mg/kg

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$$NH_2$$

SINEFUNGIN

$$HOOC-CH-CH_2-CH_2-CH-CH_2$$
$$\quad\quad NH_2 \quad\quad\quad\quad NH_2$$

OH OH
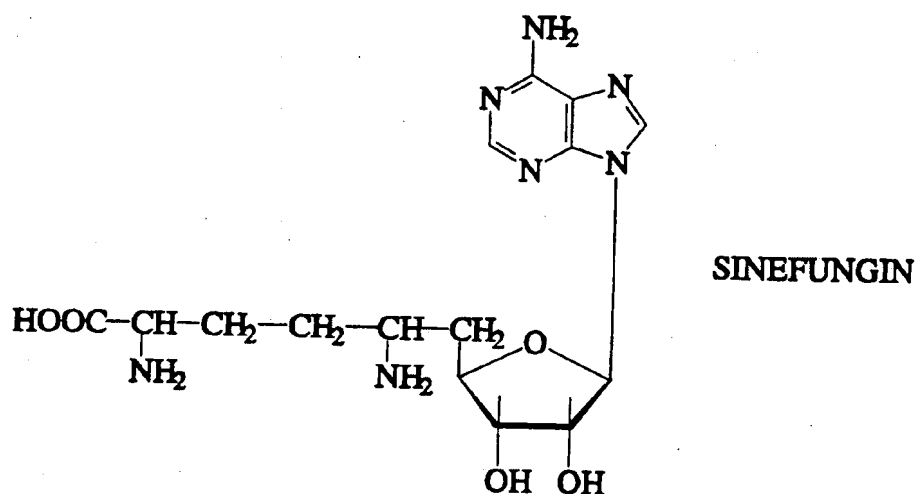
body weight/day, and preferably, between about 1 to about
50 mg/kg body weight/day, may be used in combination
with, e.g., MLS antibiotics, to kill bacteria.

5   **Adenine Derivatives**

     Moore (1970) described the differential inhibition
of tRNA methylases by adenine derivatives such as
adenine, dimethylallyladenine, isopentenyladenosine,
10   adenine phosphate, adenine sulfate, adenine-N^1-oxide,
6-methoxyadenine, 6-dimethyladenine, 6-methyladenine and
6-mercaptoadenine. It was found that all such compounds
gave inhibition that would be acceptable for the purposes
of the present invention, with the possible exceptions of
15   6-methoxyadenine and 6-dimethyladenine, and that
dimethylallyladenine and isopentenyladenosine were
particularly effective.

     Therefore, adenine, adenine phosphate, adenine
20   sulfate, adenine-N^1-oxide, 6-methyladenine and
6-mercaptoadenine may be used in combination with agents
such as MLS antibiotics at doses of between about 10 to

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about 200, and preferably, between about 25 to about 100
mg/kg body weight/day; with dimethylallyladenine and
isopentenyladenosine having proposed doses of between
about 1 to about 100, and preferably, between about 10 to
5   about 75 mg/kg body weight/day.

Nicotinamide

       Swiatek *et al.* (1973) have shown that the inhibition
10   of porcine liver tRNA methyltransferase activity by
nicotinamide is due to an active methyltransferase enzyme
that, in the presence of SAM and nicotinamide, produces
1-methylnicotinamide and SAH, the latter of which
inhibits methyl transferase enzymes.
15

       Nicotinamide (0.41 M) was evaluated by the inventors
with the first procedure and shown to enhance the
relative activity of erythromycin against *S. aureus* 27660
by 183% (Table 8). The sc. LD_{50} of nicotinamide is
20   1.68g/kg in rats (Windholz *et al.*, 1983). It is
therefore proposed that dosage of 1 mg/kg to 1.5 g/kg/day
in combination with, e.g., MLS antibiotics, will kill
bacteria and other microbes, the preferred dosage being
100-1000 mg/kg/day.
25

Methinin

       Methinin is a natural occurring methylation
inhibitor that is isolatable from rat and that inhibits a
30   number of methyltransferases. Methinin is a low
molecular weight compound (1400) that has an active amine
group and appears to be of a peptide nature (Lyon *et al.*,
1987). It inhibits DNA methylation, and has potential
for use with antimicrobial agents in the present
35   invention.

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5'-Deoxy-5'-(methylthio)adenosine (MTA)

MTA is an inhibitor of several SAM-dependent
methylations (Law et al., 1992) and, in addition, is also
5   an inhibitor of spermine and spermidine synthesis
(Yamanaka et al., 1987). Wolford et al. (1984) injected
MTA in mice ip. for 28 days. Single injections of as
high as 75 mg/kg were administered. The inventors
suggest that MTA at dosages of 1 to 200 mg/kg, and
10   preferably, of 10 to 75mg/kg/day, in combination with,
e.g., MLS antibiotics, will be effective in killing
microorganisms. MTA is commercially available from Sigma
Chemical Company.

15   Xylosyladenine (9-B-D-xylofuranosyladenine)

Garrett & Kredich (1981) showed that xylosyladenine
(9-B-D-xylofuranosyladenine) caused marked inhibition of
RNA methylation and showed that methyluridine, 2'-O-
20   methylcytidine, 5-methyluridine and 5-methylcytidine
formation in RNA was reduced. DNA methylation was not
inhibited. From data such as this, it is proposed that
xylosyladenine and other xylofuranosyl analogues of SAM
or SAH, can be used with antimicrobial agents for killing
25   microorganisms in doses of between about 0.1 to about
150 mg/kg body weight/day, and preferably, of between
about 10 to about 40 mg/kg body weight/day.

EXAMPLE V
30   <u>Inhibitors of SAH Hydrolase (SAHH)</u>

Certain Adenosine Analogues

S-adenosyl-L-homocysteine (SAH) hydrolase (SAHH) has
35   been proposed as a target for antiviral agents (Wolfe &
Borchardt, 1991). A good correlation exists between

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antiviral activity and SAHH inhibition. De Clercq &
Cools (1989) observed a linear relationship between IC_{50}
(concentration that inhibits replication by 50%) for a
series of adenosine analogues and their $logK_i$ values for
5 inhibition of murine L929 cell SAHH.

The following compounds have been described as first
generation broad-spectrum antiviral agents: 9(S)-(2,3-
dihydroxypropyl)adenine [(S)-DHPA], D-eritadine, (R,S)-3-
10 adenine-9-yl-2-hydroxypropanocic acid [(R,S)-AHPA],
adenosine (Ado) dialdehyde, 3-deazaadenosine (c^3-Ado),
aristeromycin (Ari) and neplanocin A (NPA or NpcA) (De
Clercq & Cools, 1985; 1989), the structures of which are
shown below.
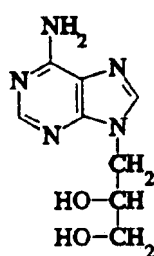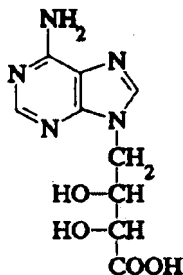
(S)-DHPA   D-Eritadenine   (RS)-AHPA   Ado dialdehyde
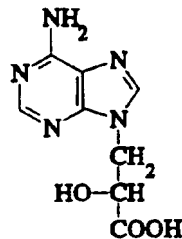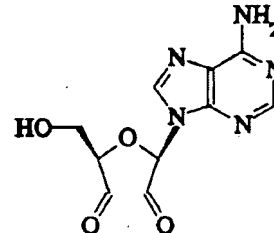
c^3-Ado   Ari   NpcA

15
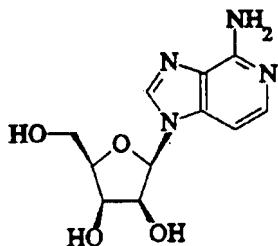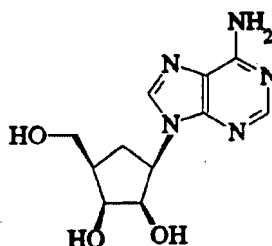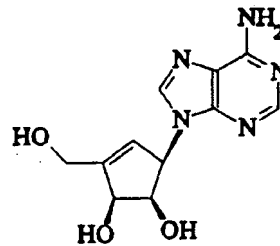
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De Clercq & Cools (1989) also indicated that
although these compounds are potent SAHH inhibitors, the
cytotoxicity of these compounds may preclude clinical use
as antiviral agents. However, since the ID_{50}/IC_{50}

5   required in the antibacterial case is contemplated to be
less stringent than that required for an antiviral agent,
the inventors contemplate that such first generation
compounds may be effectively used in the present
invention at concentrations that are not cytotoxic.

10   Additionally, these compounds could be used with
inhibitors of adenosine kinase and adenosine deaminase
(Wolfe & Bochardt, 1991) to reduce toxicity.

The ID_{50}/IC_{50} ratio required for safe clinical use of

15   the proposed inhibitor combinations with antimicrobial
agents, such as MLS antibiotics, is assessed by
determining the ID_{50} and IC_{50} experimentally using mice or
rats. The ID_{50} and IC_{50} values are determined as
described, e.g., by Cleeland & Squires (1991) and

20   Klaassen (1990).

As shown by Wolfe and bochardt (1991), SAH/SAM ratio
ranges associated with therapeutic effectiveness (IC_{50})
and cytotoxicity (ID_{50}) can be determined, e.g., using

25   HPLC. The concentrations of candidate inhibitors used
clinically in combination with antibiotics will be
determined using the IC_{50}, ID_{50} and SAH/SAM ratios as
determined above. Such concentrations are currently
contemplated to lie in the general ranges stated below.

30

The activity spectrum of (D)-eritadine is similar to
that of (S)-DPHA (Schanche et al., 1984). D-eritadine,
L-eritadenine and L-threo-eritadenine inactivate SAH in
hepatocytes. Proposed doses for use in microbial

35   killing, when combined with, e.g., MLS antibiotics, of
between about 1 to about 100 mg/kg body weight/day), and

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preferably of between about 10 to about 75 mg/kg body
weight/day, are proposed for the eritadenine derivatives.

5   De Clercq & Cools (1985) reported the inhibition of
SAH hydrolase by the following compounds at the stated
concentrations: (S)-DPHA (Ki = 1.4 μM); (RS)-AHPA
(Ki = 0.073 μM), carbocyclic 3-deazaadenosine
(Ki = 0.013 μM) and NPA (Ki = 0.002 μM). Kitaoka et al.
(1986) also showed inhibition and Backlund et al. (1986)
10   showed that N- and O-methylation of mRNA was inhibited
between 31-83% by 100 μM 3-deazaadenosine.

       Since De Clercq & Cools (1985) demonstrated a
correlation between the SAH inhibition potency and their
15   antiviral potencies, it is expected that similar effects
will be seen on the inhibition of rRNA methylation by
these compounds. Also, as (S)DHPA, (R,S)-AHPA
isobutylester, carbocyclic-3-deazaadenosine (C-c^{3}Ado) and
NPA reduce vesicular stomatitis virus-induced
20   cytopathogenicity by 50% in primary rabbit kidney cells,
with minimum inhibitor concentration of 20, 2, 0.2, 0.018
μg/ml, respectively, it is further proposed that such
compounds will operate as methylation inhibitors at
similar ratios.
25

       Kitaoka et al. (1986) studied several nucleoside
analogues known to have broad spectrum antiviral
activity, e.g., ribavirin, vidarabine (Ara A),
pyraazofurin, tubercidin, carbodine, [(S)-DHPA], (C-
30   c^{3}Ado), [(RS)-AHPA] isobutyl ester and neplanocin A.
These compared for their potency and selectivity as
inhibitors of human retrovirus replication in vitro.
These adenosine analogues were proposed to also owe their
antiviral activity to the inhibition of SAH hydrolase.
35   The studies of Kitaoka et al. (1986), reported effective
inhibitory concentrations of (S)-DPHA, C-C^{3}Ado, (RS)-AHPA

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isobutyl ester and NPA are about 60, 1.4, 1.2 and
0.2 μg/ml, respectively. *In vitro* cytotoxicity indicated
an activity index of >3, 70. 80 and >20 respectively for
(S)-DPHA), C-C^{3}Ado, (RS)-AHPA isobutyl ester and NPA,
5   suggesting a potential for *in vivo* use.

      The inventors have translated the above information
into proposed doses for use in killing microorganisms,
when combined with, e.g., MLS antibiotics, of between
10   about 0.1 to about 100 mg/kg body weight/day, and
preferably, of between about 0.1 to about 30 mg/kg/day
for (S)-DPHA, C-C^{3}Ado, (RS)-AHPA, Ari and NPA.

      Wolfe and Borchardt (1991) also described second
15   generation SAHH hydrolase inhibitors that have retained
antiviral activity and considerably lower cytotoxicity
than their parent first generation inhibitors. These
compounds include: dihydroxycyclopentenyladenine (DHCeA)
and dihydroxycyclopentenyl-3-deazaadenine (c^3-DHCeA);
20   dihydroxycyclopentanyladenine (DHCaA) and
dihydroxycyclopentanyl-3-deazaadenine (c^3-DHCaA);
3-deazaneplanocin A(c^3-NpcA) and 3-deazaaristeromycin
(c^3-Ari), as shown below.

- 106 -
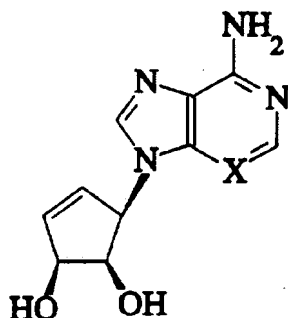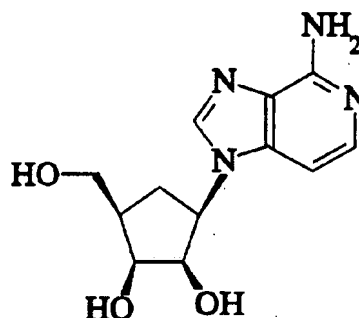

DHCeA: X = N
c³-DHCeA: X = CH
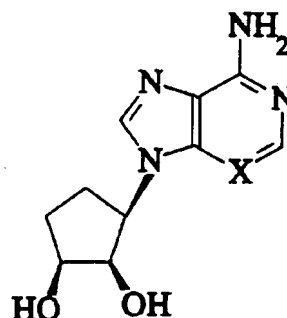
DHCaA: X = N
c³-DHCaA: X = CH
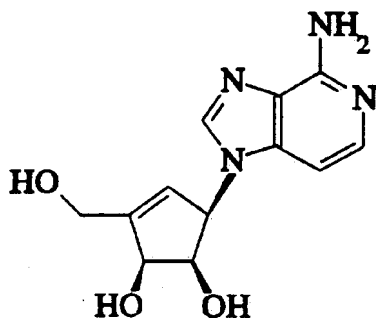
c³-NpcA

Ari

The increased therapeutic effectiveness of the second generation inhibitors (ratio of ID_{50}/IC_{50}) is apparently due to the elimination of adenosine kinase and adenosine deaminase activity of these generation

5   compounds. It is contemplated that the second generation inhibitors will function successfully in clinical combinations with antimicrobial agents, as exemplified by MLS antibiotics. The safe clinical dose for the second generation inhibitors, when used in combination with,

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e.g., MLS antibiotics, will be higher than for the first generation inhibitors.

Using published information, such as that in De Clerq et al. (1989), the following doses are contemplated for using the second generation compounds in combination with antibiotics to achieve microbial killing: between about 0.1 and about 100; preferably, between about 0.1 and about 50; and most preferably, between about 0.1 and about 10 mg/kg body weight/day for DHCeA, c^3-DHCeA, DHCaA, c^3-DHCaA, A(c^3-NpcA) and c^3-Ari.

2'-deoxy-2'-chloroadenosine (CLA) was shown to be an inhibitor of hamster and human liver SAHH (Kim et al., 1985). Buckle et al. (1981) evaluated CLA at dosages of 300 μMol/kg (90 mg/kg) in mice. Based on these observations, it is proposed that CLA in combination with, e.g., MLS antibiotics, will provide enhanced killing at dosages between 1 and 150 mg/kg/day, and preferably, at between 10-90 mg/kg/day.

Methylthioadenosine (MTA, 0.042 M) and 2-Chlorodeoxyadenosine (0.035 M) were evaluated by the inventors with the first procedure and shown to enhance the relative activity of erythromycin against S. aureus 27660 by 183% and 163% respectively (Table 8). MTA is thus also proposed for clinical use with, e.g., MLS antibiotics, at dosages of between 1 and 150 mg/kg/day.

6-(γ,γ-Dimethylallylamino)purine riboside 1-200. 10-100 6-(γ,γ-Dimethylallylamino)purine riboside is commercially available from Sigma Chemical Company.

6-(Isopentenyl)adenosine 1-200. 10-100 6-(Isopentenyl)adenosine is commercially available from Sigma Chemical Company.

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6-Dimethylaminopurine riboside 1-200.   10-100
6-Dimethylaminopurine riboside is commercially available
from Sigma Chemical Company.

5   **Neplanocin A (NPA)**

Neplanocin A (NPA) is a cyclopentenyl analogue of
adenosine that is a potent inhibitor of SAH hydrolase.
NPA has been shown to inhibit vaccinia virus
10   multiplication in mouse L929 cells. Concentrations of
NPA as low as 0.5 and 1 μM in the culture medium have
been reported to produce 84 and 95% inhibition of plaque
formation, respectively, while exhibiting little toxicity
to the host cells (Borchardt et al., 1984). This finding
15   suggests that the antiviral action of this compound is
related to an inhibition of SAM-dependent methylation
reactions that are essential to the production of new
viral particles (e.g. viral messenger RNA).

20   Yaginuma and colleagues reported that NPA possesses
significant antitumor activity in vivo (Yaginuma et al.,
1979; 1981; Tsujino et al., 1979). This indicates that
NPA is safe for in vivo use.

25   Glazer & Knode (1984) also studied the activity of
NPA against human colon carcinoma in vitro. They
determined NPA to be an inhibitor of RNA methylation, but
not of RNA synthesis (transcription). These properties
confer to NPA unique pharmacological characteristics not
30   possessed by other adenosine analogues studied at that
time. Consequently, NPA should be a valuable tool for
further chemotherapeutic studies.

NPA is available from the Toyo Jozo Co., Ltd., Japan
35   and it may be synthesized according to the methods of Lim
& Marquez (1983). From the studies showing that NPA

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inhibited malarial growth with ED_{50} of $3\mu M$ (Whaun
et. al., 1986), and because NPA does not cause cellular
toxicity ((Yaginuma et al., 1979; 1981; Tsujino et al.,
1979), it is contemplated that useful concentrations of
5   NPA for use in killing microorganisms (in combination
with antimicrobial agents) will be from about 1 to about
50 mg/kg body weight/day, and preferably, from about 1 to
about 20 mg/kg body weight/day.

10   The inventors make this proposal despite certain
prior teachings concerning NPA. For example, Fischer et
al. (1987) studied the effect of Neplanocin A on sixteen
bacteria cultured on agar plates, and only two were
determined to be sensitive. None of the sixteen showed
15   any growth sensitivity in broth culture to concentrations
as high as 4 mM (Fischer et al., 1987). The authors
concluded that SAH as a target for antimicrobial agent
development seemed not likely to be a productive approach
(Fischer et al., 1987).
20

         In marked contrast to the teachings represented by
Fischer et al. (1987), the present inventors have
unexpectedly found that several SAH inhibitors very
similar to Neplanocin A were effective in enhancing the
25   action of MLS antibiotics. In particular, Ara-A, 3-
Deazaneplanocin A, methylthioadenosine and 2'-
deoxyadenosine, used in combination with erythromycin,
enhanced the killing of resistant S. aureus 27660
bacteria (Table 8).
30

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3-deazaneplanocin A (c^3-NPA)

Tseng *et al.* (1989) described the synthesis of the
neplanocin A analogue 3-deazaneplanocin A (also
5   obtainable from Dr. V.E. Marguez; National Institutes of
Health, Bethesda, Maryland). This derivative is neither
phosphorylated nor converted to the
S-nucleosidylhomocysteine analogue and is therefore
significantly less cytotoxic than neplanocin A.
10   3-deazaneplanocin A is thus contemplated to be one of the
preferred compounds for use in combination with one or
more MLS antibiotics or other antimicrobial agents.

Glazer *et al.* (1986) showed that NPA (10 μM), c^3-NPA
15   (10 μM) and c^3-Ari (100 μM) inhibit the methylation of
28S rRNA, 18S rRNA and tRNA. Additionally it was shown
that NPA, c^3-NPA and c^3-Ari showed cytotoxicity of HT-29
cells above 1, 100 and 250 μM, respectively. It is
therefore expected that c^3-NPA and c^3-Ari could be used
20   at dosages 10-100 times higher than that of NPA, i.e.,
used (in combination with antibiotics) at doses of from
about 0.1 to about 1,000 mg/kg body weight/day, and
preferably, from about 0.1 to about 200 mg/kg body
weight/day.
25

As a practical matter, c^3-NPA (0.031 M; from Dr.
V.E. Marguez) was evaluated by the inventors using the
first disc susceptibility test, when it was shown to
enhance the activity of erythromycin against *S. aureus* by
30   167% (Table 8).

Other Neplanocin A Analogues

Shuto *et al.* (1992) synthesized a number of 6'-
35   modified Neplanocin A derivatives, which were evaluated
for their SAHH and antiviral activities. Each of the

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compounds described were particularly active against a
number of viruses. Additionally, they showed increased
selectivity with 6'-C-methylneplanocin A surpassing
neplanocin A both in antiviral potency and selectivity.

6'-C-methylneplanocin A, virtually resistant to
adenosine deaminase and a good inhibitor of murine L929
SAHH, is expected to function extremely well in
combination with MLS antibiotics. It is contemplated
that this agent may be used in combination with, e.g.,
MLS antibiotics, at doses of from about 1 to about 200
mg/kg body weight/day, and preferably, from about 1 to
about 100 mg/kg body weight/day.

Ara-A and Arabinoside Compounds

2'-Deoxyadenosine and 9-B-arabinofuranosyladenine
(Ara-A) are suicide inactivators of SAH hydrolase
(Hershfield, 1979; Helland & Ueland, 1982). Ara-A
appears to be twice as active as 2'-deoxyadenosine. Ara-
A is approved by the FDA for use as an ophthalmic
ointment for herpes simplex virus infections and also as
a systemic treatment for herpes simplex encephalitis at a
dosage of 15 mg/kg/day for 10 days (Berkow et al., 1992).
This suggested to the inventors that Ara-A could be used
therapeutically.

Ara A (0.039 M) and 2'-deoxyadenosine (0.183 M) were
evaluated by the inventors using the first disc
susceptibility test, and shown to enhance the relative
erythromycin activity against the resistant S. aureus
27660 by 193 and 233% respectively (Table 8). The
proposed dosages for Ara-A and 2'-deoxyadenosine clinical
use range between about 1-300 mg/kg/day, and preferably,
between about 1-100 mg/kg/day. LD_{50} of Ara-A in mice is

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4,677 mg/kg (i.p) and 7,950 mg/kg orally (Windholz
et al., 1983).

5   Although Sacks et al. (1982) have suggested that the
inactivation of SAH hydrolase could mediate toxic
manifestations of ara-A therapy in certain group of
patients, used at the appropriate therapeutic levels,
Ara-A is contemplated to be an effective agent for
combination with antibiotics. Both Ara-A and
10   2-Deoxyadenosine are commercially available from Sigma
Chemical Company (St. Louis, MO, USA) and Parke-Davis &
Co. (Detroit).

Aristeromycin (9-β-{2,3-dihydroxy-4-(hydroxymethyl-
15   **cyclopentyl}adenine) and Analogues**

Houston et al. (1985b) synthesized a series of
purine (adenine, N^6-methyladenine, 8-azaadenine, 3-
deazaadenine) carbocyclic nucleosides, nucleoside-2',3'-
20   dialdehydes and nucleoside-2',3'-diols as potential
inhibitors of SAHH and vaccinia virus replication. They
observed a good correlation between a compound's
inhibitory effect on SAHH and its antiviral effects,
suggesting that viral inhibition is caused by the
25   inhibition of a critical methylation step. Similar
antimicrobial activity is expected from these compounds.

The most effective inhibitors of bovine liver SAHH
from the synthesized series were evaluated at 0.2 mM
30   Aristeromycin (99% inhibition), N^6-methylaristeromycin
(22%), 8-azaaristeromycin (46%) and 3-deazaaristeromycin
(97%). The inhibition of the corresponding dialdehyde
derivatives at 0.2 mM ranged from 32 to 100%, while the
corresponding diol derivatives showed between 82 to 98%
35   inhibition only at the 2 mM level. The % inhibition of
vaccinia plaque formation by the compounds above at the

- 113 -

10 μM level followed to a large extent the ability to inhibit SAHH. Similarly, (Keller & Borchardt, 1987) showed that the dialdehyde derivative produces an increase in intracellular levels of SAH and subsequent
5   inhibition of SAM-dependent methylations.

(±)-5-Noraristeromycin and its 2,6-diamino-analogue were synthesized (Patil & Schneller, 1992; structures shown below) and shown to be inhibitors of SAHH. In
10   antiviral evaluations it was observed that (±)-5-Noraristeromycin was 10 to 20 times less cytotoxic in cell culture. It is therefore expected that both these compounds will function well in combination with MLS antibiotics.
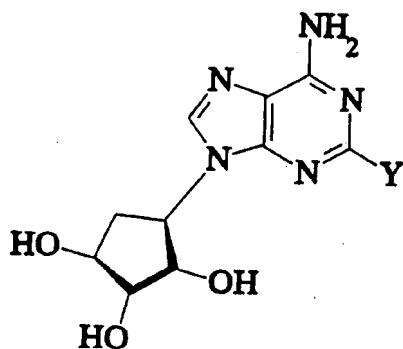
15

Y = H or NH$_2$

For aristeromycin and derivatives, the following doses are proposed for use in killing microorganisms in
30   combination with antibiotics: between about 1 to about 200, and preferably, between about 10 to about 100 mg/kg body weight/day for aristeromycin, 3-deazaaristeromycin, (±)-5-Noraristeromycin and its 2,6-diamino-analogue; between about 1 to about 100, and preferably, between
35   about 5 to about 50 mg/kg body weight/day for N^6-methylaristeromycin; and between about 5 to about 200,

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and preferably, between about 10 to about 100 mg/kg body
weight/day for 8-azaaristeromycin.

One of the cellular reactions affected by
aristeromycin is transmethylation. Because of the
sensitivity of the transmethylases to the
S-aristeromycinyl analog of SAH, it is likely that the
eukaryotic mRNAs or viral mRNA methyltransferases will be
inhibited. Due to its cytotoxicity to mammalian cells,
many derivatives have been synthesized to overcome
toxicity. These include 2'-deoxy-, 3'-deoxy-, 3'-amino-
3'-deoxy-, 3'-amino-3'-deoxyarabinofuranosyl, 6'-hydroxy,
6'-mercapto, 8'-bromo, 8-hydroxyaristeromycin,
aristeromycin-3'-cyclic phosphate and aristeromycin-6'-
cyclic phosphate. The appropriate doses for any given
aristeromycin derivative will lie in the ranges described
above, closest to the structural analogue which the
derivative most closely resembles. Optimization of the
dose will be routine given these guidelines.

The structure of aristeromycin is shown below, it is
commercially available from Sigma Chemical Company.
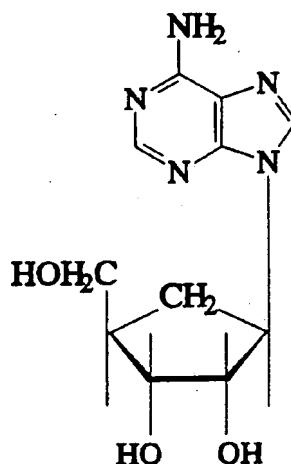
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Tubercidin (7-deazaadenosine)

Enzymes that methylate tRNA are known to have qualitative and quantitative differences among different species. Inhibitors of these enzymes with three different specificities have been identified, namely some restricted bacterial enzymes, some to mammalian enzymes, and others with dual potencies (Wainfan & Borek, 1967). Adenine, adenosine, isopentenyladenosine and tubercidin are inhibitory to tRNA methylases isolated from *E. coli* and thus inhibit methylation.

As tubercidin inhibits SAHH, the SAH/SAM ratio increases and inhibits methylation. This is one instance of a compound that exerts an effect at various points in the cell. Tubercidin and isopentenyladenosine are commercially available from Sigma Chemical Company. Tubercidin has the following structure:
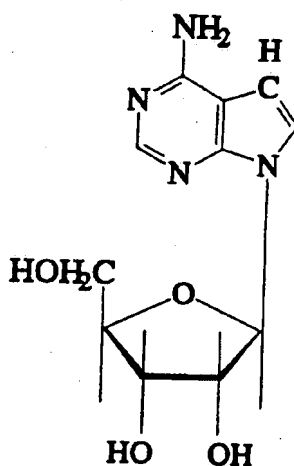
Tubercidin also inhibits rRNA processing in addition to methylation of tRNA and protein and nucleic acid synthesis. The substitution of tubercidin for adenosine in SAH yields a potent inhibitor of tRNA methylase,

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Catechol-O-methyltransferase, indole ethylamine
N-methyltransferase, methylation of tRNA in
phytohemagglutinin-stimulated lymphocytes and polyamine
biosynthesis.

5

Tubercidin (0.034 M) was evaluated by the inventors
with the first procedure and shown to enhance the
relative activity of erythromycin against *S. aureus* by
193% (Table 8). The LD_{50} of tubercidin iv. in mice is 45
10   mg/kg (Windholz *et al.*, 1983). Using this data, the
inventors propose the following tubercidin dosage range
for use with, e.g., MLS antibiotics, in killing bacteria:
between about 0.1 to about 40, and preferably, between
about 1 to about 20 mg/kg body weight/day.

15

EXAMPLE VI

Inhibitors of SAM Synthetase (SAMS)

20   **Cycloleucine**

Cycloleucine (1-aminocyclopentane-1-carboxylic acid)
is an inhibitor of SAM synthetase (SAMS, or methionine-
adenosyltransferase). Finkel & Groner (1983) reported
25   that cycloleucine, at 0.5 mg/ml, causes more than a 30%
decrease in internal m^6A of late SV40 mRNA, with only a
minor effect on the dimethyladenosine of the 5' cap
structure. After treatment with 2 and 5 mg/ml
cycloleucine, internal m^6As were reduced by 10- and
30   100-fold, respectively.

Kroes *et al.* (1984) have shown that nitrous oxide
and cycloleucine have synergistic growth inhibiting
effects in experimental rat leukemia (BNML). These
35   results indicated that a reduction of SAM synthesis by
cycloleucine can increase the disturbance of folate

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metabolism that is caused by nitrous oxide, with a potentiation of the effects on leukemic growth.

Cycloleucine at a concentration of 0.1 M, pre-mixed
5   with Brain Heart Infusion Agar, showed improved killing
(240% increase over control) of *S. aureus* resistant to
erythromycin (FIG. 2). From the above inhibitory range
of 0.5 to 5 mg/ml and the LD_{50} of 309 mg/kg in mice
(Windholz *et al.*, 1983), it is contemplated that
10   therapeutic cycloleucine doses of between about 1 and
about 250 mg/kg body weight/day, and preferably, of
between about 10 and about 50 mg/kg body weight/day, will
be of use in combination with antibiotics to treat
infections. Cycloleucine is commercially available from
15   Sigma Chemical Company.

Methionine Analogues

Sufrin *et al.* (1979) reported that mono-, bi- and
20   tri-cyclic amino acid structural and conformational
analogues of L-methionine functioned as inhibitors of the
enzymatic synthesis of S-adenosyl-L-methionine. These
synthetic amino acids are structurally and
conformationally related to cycloleucine (described
25   above). Compounds that function as effective inhibitors
include cyclopentaneglycine, the (*1R,2R,4S*) isomer of
2-aminonorbornane-2-carboxylic acid, the (*1R,2R,4S*)
isomer of 2-amino-5,6-*exo*-trimethylenenorbornane-2-
carboxylic acid, 3-aminobicyclo[3.2.0]heptane-3-
30   carboxylic acid and, most effective, (+)-2-
aminobicyclo[2.1.1]hexane-2-carboxylic acid.

The concentration for 50% inhibition of these
compounds are similar to that of cycloleucine. It is
35   therefore expected that similar concentration as those

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contemplated for cycloleucine will be therapeutically active in combination with, e.g., MLS antibiotics.

Lombardini & Sufrin (1983) described the chemotherapeutic potential of methionine analogue inhibitors of tumor-derived methionine adenosyltransferases. In addition to cycloleucine, 1-aminocyclobutanecarboxylic acid, 1-aminocyclohexanecarboxylic acid, L-2-amino-4-hexanoic acid, (Z)-L-2-amino-5-chloro-trans-4-hexanoic acid, L-ethionine, and seleno-DL-ethionine were found to be effective inhibitors, with (+)-2-aminobicyclo[2.1.1]hexane-2-carboxylic acid again being found to be the most effective. Since the I_{50} of these compound are similar to that of cycloleucine, similar therapeutic performance is expected.

Kramer et al. (1987) described the relative effects of SAM depletion on nucleic acid methylation and polyamine biosynthesis. It was shown that the treatment of cultured L1210 cells with 1 mM L-2-amino-4-methoxy-cis-but-3-enoic acid (L-cis-AMB), a methionine analogue inhibitor of SAM synthetase, produced a rapid and near-total depletion of SAM by 4 hours. Kramer et al. (1988) also reported that L-cis-AMB, the most potent methionine-analogue inhibitor of SAM synthetase at that time, is capable of rapidly depleting SAM pools in intact cells without affecting precursor incorporation into macromolecules.

Growth inhibition in L1210 cell culture cells by methionine analogue inhibitors of SAM biosynthesis in the absence of polyamine depletion has been reported by Porter et al. (1984). In addition to cycloleucine (IC_{50}=5 mM) and (+)-2-aminobicyclo[2.1.1]hexane-2-carboxylic acid (IC_{50} = 5 mM), this group identified

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selenomethionine (IC_{50} = 0.13 mM) and L-*cis*-AMB
(IC_{50} = 0.4 mM) as effective inhibitors, with L-*cis*-AMB
being more potent. The IC_{50} of L-*cis*-AMB is about 10
fold lower than that of cycloleucine: it is therefore
expected that it will operate therapeutically at lower
concentration in combination with, e.g., MLS antibiotics.

Sufrin *et al*. (1993) later described the activity of
L-2-amino-4-methylthio-*cis*-but-3-enoic acid (L-*cis*AMTB)
as an inhibitor of SAM-synthetase. L-*cis*-AMTB was
slightly less inhibitory than L-*cis*-AMB, but is
nonetheless effective. The structures of L-*cis*-AMB and
L-*cis*-AMTB are shown below:

L-cis-AMB   L-cis-AMTB
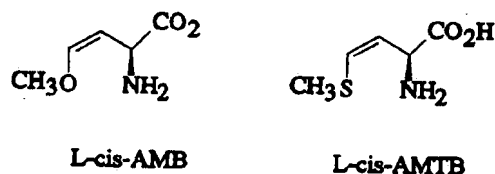
For the methionine analogues, it is contemplated
that concentrations generally around 1-150 mg/kg body
weight/day may be used in combination with antimicrobial
agents to kill bacteria and other microbes. More
specifically, for (+)-2-aminobicyclo[2.1.1]hexane-2-
carboxylic acid, a preferred therapeutic dose of between
about 10 and about 50 mg/kg body weight/day is proposed;
and for L-*cis*-AMB, a therapeutic dose of between about 1
and about 100 mg/kg body weight/day, and preferably, of
between about 5 and about 50 mg/kg body weight/day is
contemplated.

ATP Analogues

Ma *et al*. (1990) described a family of ATP analogues
that are either mono- or di-substituted with imido and

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methylene bridges in the polyphosphate chain: adenosine
5'-(α,β:β,γ-diimidotriphosphate) (AMPNPNP), adenosine
5'-(α,β:αβ',-diimidotriphosphate) (AMP(NP)₂), and
adenosine 5'-(α,β:β,γ-dimethylenetriphosphate) (AMPCPCP).

5   These were investigated as substrates and inhibitors of
SAM synthetase. AMPNPP was found to be both a substrate
for SAM synthetase and a potent inhibitor, with an
inhibition constant 60 fold less than the K_m of ATP.
AMPNPP was about 1000-fold more effective as an inhibitor

10   than AMPCPP.

The synthesis of AMPCPP and the other analogues is
described in Ma *et al.* (1990), the structure of which is
depicted below. Since the K_i value for AMPCPP is similar

15   to that of cycloleucine, equivalent therapeutic potency
is expected.

20
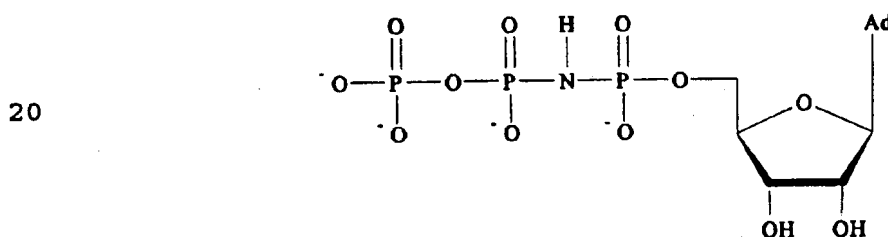
25

Further Inhibitors

Numerous inhibitors of SAM synthetase have been
synthesized in the laboratory of Kappler. Their

30   synthesis and biological activity have been reported in a
number of papers (Kappler *et al.*, 1986a; 1986b; 1987;
1988; Lim *et al.*, 1986; Vrudhula *et al.*, 1987; 1989).

Vrudhula *et al.* (1989) reported the synthesis of

35   nonselective inhibitors of various isozymes of rat SAM
synthetase. Four types of covalent methionine-ATP

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adducts were described to be potent inhibitors of the M-1
and M-2 isozymes. The most effective inhibitor is the
5'R epimer of a CH$_3$-C5' adduct of L-methionine and
β,γ-imido-ATP. This compound,

5 5'-(R)-(C)-[(L-homocysteine-S-yl)methyl]adenosine
5'-(β,γ-imidotriphosphate), is also described by Kappler
et al. (1987).

Inhibitors of Glutathione Synthetase

10

 Corrales et al. (1991) showed that there is a close
correlation between the hepatic levels of glutathione and
SAM synthetase activity. These authors suggested that
under normal conditions, oxidation of cysteine groups of
15 SAM synthetase may be protected by the intra-cellular
levels of glutathione. The SAM-synthetase activity of
animals treated with buthionine sulfoximine was shown to
be only about 40% of that of untreated animals.

20 DL-buthionine-(S,R)-sulfoximine or L-
buthionine(S,R)-sulfoximine used in most studies contains
4 or 2 isomers, respectively (Campbell et al., 1991).
These authors showed that the different isomers were
effective in reducing GSH levels in mice liver, kidney
25 and pancreas at the concentrations of 0.2 and 0.4 mmol/kg
respectively with L-buthionine-(S)-sulfoximine and
L-buthionine-(S,R)-sulfoximine. L-buthionine-(R)-
sulfoximine did not show any significant depletion of GSH
at 0.2 mg/kg in liver or pancreas, but modest depletion
30 in the kidney.

 Lasierra et al. (1989) determined that buthionine
sulfoximine [4.5 mmol/kg (1g/kg) and diethyl maleate (3.2
mmol/kg) (0.55 g/kg)] ip. depleted liver and aortic
35 glutathione in rabbits. Muzutani et al. (1994) suggested

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the use of buthionine sulfoximine could have application
in tumor therapy.

As inhibitors of glutathione synthetase decrease the
activity of SAM-synthetase, the present inventors thus
propose the use of any glutathione synthetase inhibitors
in combination with antibiotics. One such example is the
buthionine sulfoximine mentioned above. Buthionine
sulfoximine has the structure:
$CH_3(CH_2)_2S(O)(=NH)CH_2CH_2CH(NH_2)COOH$, and is commercially
available from Sigma Chemical Company.

Based on the *in vivo* data presented by Corrales *et
al.* (1991), and on the fact that the GSH levels can be
depleted in a dosage dependent fashion (Drew *et al.*,
1984), the inventors contemplate that suitable
therapeutic doses of buthionine sulfoximine will be
between about 1 and about 1000 mg/kg body weight/day, and
preferably, of between about 1 and about 100 mg/kg body
weight/day. The inventors have shown that when
L-buthionine(S,R)-sulfoximine (0.09 M) was evaluated in a
disc susceptibility assay it enhanced the relative
activity of erythromycin against *S. aureus* by 167% (Table
8).

Many other examples of glutathione synthetase
inhibitors have been described, any one or more of which
may be used in connection with the present invention.
Kato *et al.* (1987) showed sequence homology of over 40
amino acids between glutathione synthetase of *E. coli* and
dihydrofolate reductase from *E. coli* and mammalian
sources. It was shown that *E. coli* glutathione
synthetase was potently inhibited by 7,8-dihydrofolate,
methotrexate and trimethoprim. α-aminomethylglutarate
was described by Sekura *et al.* (1976) as an inhibitor of
glutathione synthetase. SAPH-3 disulfide was shown to be

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a potent inhibitor of glutathione synthetase (Schor et al., 1991). Suitable doses of 7,8-dihydrofolate, trimethoprim, α-aminomethylglutarate and SAPH-3, for use in combination with antibiotics, will generally be
5   between about 0.1 and about 200 mg/kg body weight/day, and preferably, between about 1.0 and about 75 mg/kg body weight/day.

10   **EXAMPLE VII**
                Inhibitors of Methionine Synthetase
                and Homocysteine Transmethylase

Nitrous Oxide (N₂O)
15

     Yagiela (1991) reported that nitrous oxide reacts with the reduced form of vitamin B_{12}, consequently inhibiting methionine synthetase, an enzyme that directly supports methylation reaction and nucleic acid synthesis.
20   N_2O is commercially available from Aldrich Chemical Company, amongst other sources.

     50% N_2O is usually used clinically. However, evidence exists that prolonged exposure to clinical
25   concentrations of N_2O inhibits cellular proliferation and can lead to megaloblastic anemia, leukopenia, and thrombocytopenia (Yagiela, 1991). Continuous administration beyond 24 hours, or repeated administration more frequently than once every 3 to 4
30   days, should therefore be avoided to prevent leukopenia and megaloblastic changes. Biochemical recovery from exposure begins once inhalation is stopped, and can be improved by folinic acid and analogues, methionine and vitamin B_{12}. For example, Ostreicher (1994) recently
35   reported that vitamin B_{12} supplements reverse some of the effects of chronic N_2O exposure.

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It has been shown the certain bacteria, e.g.,
Salmonella typhimurium (Baden & Monk, 1981),
Streptococcus faecalis, E. coli, and *S. aureus* are
inhibited by N_2O at elevated pressures. It is therefore
5   expected that N_2O, at currently used clinical levels and
durations (as above), in combination with, e.g., an MLS
antibiotic, will show increase antimicrobial killing.
Alternatively N_2O may be used at elevated pressure in
combination with antibiotics.

10

EXAMPLE VIII
Inhibitors of Adenosine Deaminase (ADA)
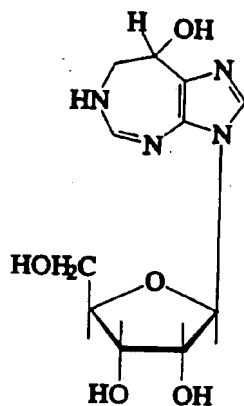
Coformycin and Isomers

15

Adenosine deaminase inhibitors are also known to
inhibit methylation indirectly by increasing the
concentration of SAH. Coformycin and its 2'-deoxy isomer
are naturally occurring potent inhibitors of ADA. Two
20   further, chemically synthesized inhibitors of ADA are 1,
6-dihydro-6-hydroxy-methylpurine nucleoside and
erythro-9-(2-hydroxy-3-nonyl)adenine (Suhadolnik, 1979).
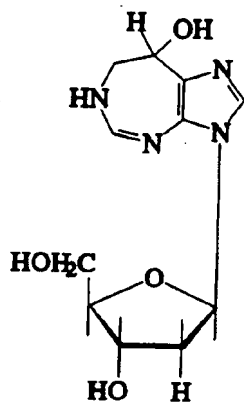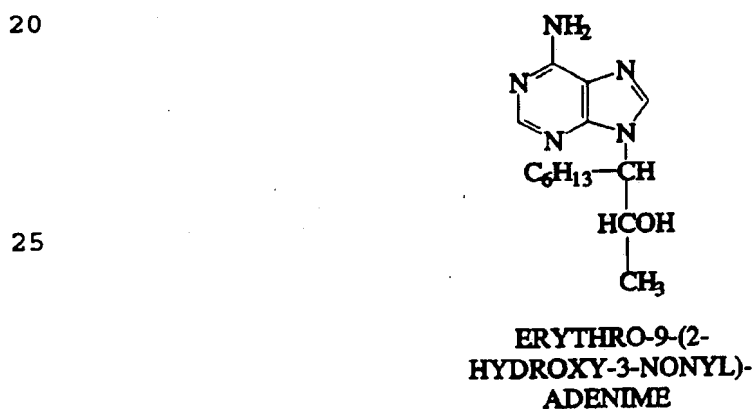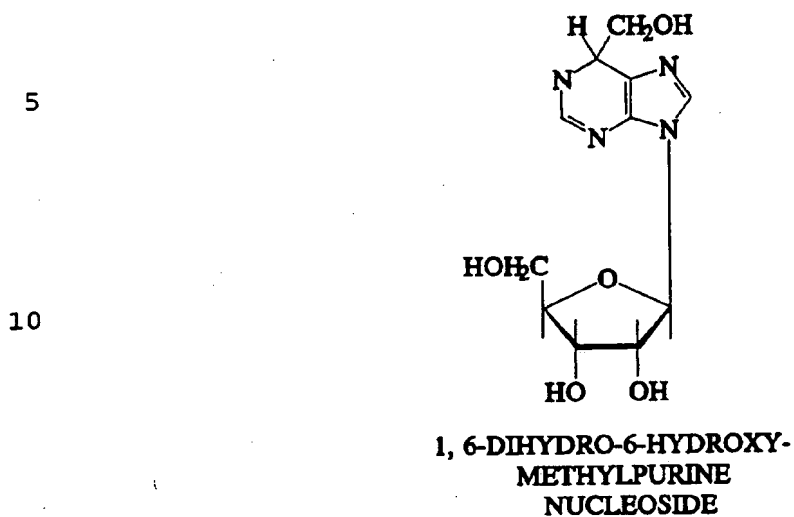These four ADA inhibitors are shown below:

- 125 -

COFORMYCIN

DEOXYCOFORMYCIN

(CO-VIDARABINE,
PENTOSTATIN)

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5

H CH₂OH

1, 6-DIHYDRO-6-HYDROXY-
METHYLPURINE
NUCLEOSIDE

10

15

20

ERYTHRO-9-(2-
HYDROXY-3-NONYL)-
ADENIME

25

30

ADA inhibitors show anti-tumor and immunosuppression
activities (Suhadolnik, 1979). Lindley & Pisoni (1993)
showed that conformycin (2.5 mM), deoxyconformycin
(0.02 mM), 2'-deoxyadenosine (2.5 mM),

35   6-methylaminopurine riboside (2.5 mM),

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2'-3'-isopropylidene-adenosine (2.5 mM) and
erythro-9-(2-hydroxy-3-nonyl)adenine (0.2 mM) inhibited
lysosomal ADA by > 97%. O'Dweyer et al. (1988) showed
that 2'-deoxyconformycin (pentostatin), has striking
5   antitumor activity. Pharmacological studies by these
investigators led to the definition of a safe and
effective low weekly dose, at which protracted ADA
inhibition occurs in neoplastic cells. This dose is 0.13
mg/kg. They also reported that patients tolerated 1
10   mg/kg/day for 5 days with minimal distress. Brogden et
al. (1993) determined the intravenous therapeutic dosage
of pentostatin for lymphoblastic leukemia to be between
0.1 to 1 mg/kg/day.

15   Pentostatin has received FDA labeling approval for
the treatment (3-6 month) of hairy cell leukemia in
combination with alpha interferon (Kane et al., 1992).
Based on these results, it is expected that dosages
similar to the approved clinical dosages of pentostatin,
20   namely 0.1 to 1 mg/kg/day, in combination with the
antibiotics, will effectively kill bacteria.

EXAMPLE IX
Inhibitors of Dihydrofolate Reductase (DHFR)
25

Methotrexate (MTX)

Nesher et al. (1991) showed that, in vitro, low
concentrations of methotrexate (MTX) interfere with
30   specific methylation reactions in peripheral blood
monocytes.

Svardal et al. (1988) suggested that MTX affects SAH
metabolism in both cultured cells and patients and this
35   may be explained by a lack of the 5-

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methyltetrahydrofolate required for salvage of SAH to
methionine.

5   MTX is used clinically at dosages of either 2.5 to
5.0 mg/day or 25 to 50 mg/single dose/week (Berkow et
al., 1992) and it is polyglutamated in vivo. It is
proposed that MTX at such clinically relevant dosages in
combination with antibiotics will kill bacteria. It was
shown by the inventors that when MTX (0.018 mM) was
10   evaluated with the first procedure it was shown to
enhance the relative activity of erythromycin against
S. aureus 27660 by 233% (Table 8).

It is also contemplated that aminopterin could be
15   used similarly to MTX.

Trimethoprim

Roth (1986) indicated that trimethoprim, which
20   retains only the 2,4-diaminopyrimidine moiety of MTX,
retains high inhibitory activity only for bacterial DHFR.
This inhibition therefore prevents tetrahydrofolate-
dependent transmethylation reactions in bacteria (Schmidt
et al., 1977), which is an important finding. From
25   results such as these the inventors propose that
trimethoprim and analogues could be used in combination
with, e.g., MLS antibiotics, to kill bacteria. The
typical clinical trimethoprim dosage is about 100 mg/12
hr (Berkow et al., 1992), and similar doses are proposed
30   for use with the present invention. The inventors have
shown in FIG. 2 that trimethoprim (0.26 mM) pre-mixed
with agar inhibits S. aureus 27660 (resistant strain)
275% relative to control.

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## 4,6-Diamino-2,2-dimethyl-s-triazines

Numerous 4,6-Diamino-2,2-dimethyl-s-triazines
analogues have been synthesized especially in the
5   laboratory of Baker (Baker & Vermeulen, 1969, 1970a,
1970b) and shown to be excellent inhibitors of DHFR. One
such analogue was evaluated by Corbett et al. (1982) as
being highly active against four transplantable colon
adenocarcinomas in mice.

10

Based on these results it is expected that 3-chloro-
4-{4-(2-chloro-4-[4,6-diamino-2,2-dimethyl-s-triazin-
1(2H)-yl)]butyl}benzenesulfonyl chloride (NSC 127755,
shown below) and 3-chloro-4-{4-(2-chloro-4-[4,6-diamino-
15   2,2-dimethyl-s-triazin-1(2H)-yl)phenoxy]methyl}-N,N-
dimethylbenzamide (NSC 139105, shown below) in
combination with antimicrobial agents such as MLS
antibiotics will kill microorganisms. Since the s.c.
toxicity in mice was determined as $\geq$ 156 mg/kg (LD_{100}),
20   it is expected that NSC 127755 and analogues will be used
at concentrations of about 1 to 100 mg/kg body weight and
preferably at about 1 to 50 mg/kg.
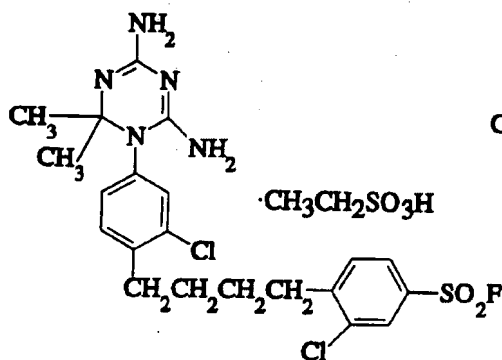

NSC 127755
TRIAZINE ANTIFOLATE
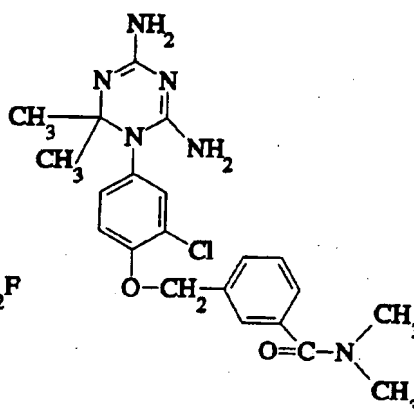
NSC 139105
BAKER'S ANTIFOL

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2,4-Diamino-5-(3,4-dichlorophenyl)pyrimidines

Baker & Vermeulen (1969; 1970b) also described
inhibitors of this group. It is expected that inhibitors
of this group in combination with MLS antibiotics will
kill microorganisms at dosages similar to that of the
4,6-diamino-2,2-dimethyl-s-triazines described above.
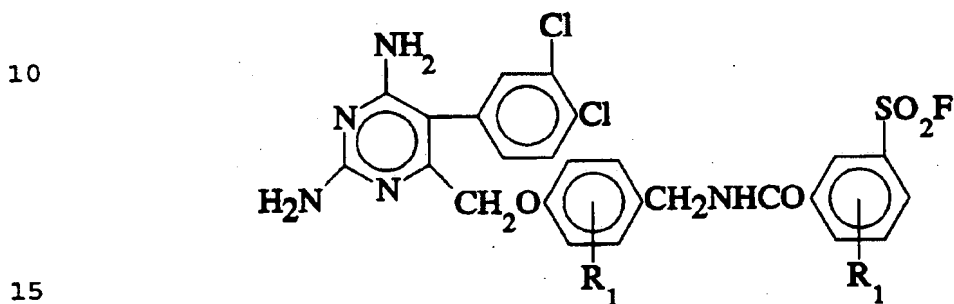
$$R_1 = R_2 = H$$
$$R_1 = H;\ R_2 \neq H$$
$$R_1 \neq H;\ R_2 = H$$

Further Inhibitors

Numerous inhibitors of DHFR are known in the
literature (Roth, 1986; Bowden et al., 1993; Fleming &
Schilsky, 1992). Blaney et al. (1984) reviewed over 1700
inhibitors of DHFR known at that time. To compare the
inhibitors synthesized in numerous laboratories, these
authors have reported the results in terms of either log
$1/C$ or log K_{iapp}. The potency of inhibitors were
calculated from reported I_{50} or K_i values and expressed
as either log $1/C$ or log K_{iapp}. No distinction was made
between these two parameters in this publication. The
semi-quantitative nature of these parameters are suitable
to indicate the approximate potency of the reported

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preferably >5, will work in combination with antibiotics
to kill bacteria and microorganisms.

Such inhibitors are contemplated for use in
combination with antibiotics at doses of between about
0.1 and about 200 mg/kg body weight/day, and preferably,
between about 1 and about 100 mg/kg body weight/day.

Inhibitors of Dihydropteroate Synthetase: Sulfonamides

Bacteria cannot utilize preformed folic acid.
Sulfonamides function as competitive inhibitors of p-
aminobenzoic acid (PABA) incorporation into
dihydropteroic acid, the immediate precursor of folic
acid. Sensitive organisms are those that must synthesize
their own folic acid. Mammalian cells are not effected
since they required preformed folic acid. In addition,
sulfonamides combine with the pteridine moiety to deplete
the system of pteridine if PABA is not present (Madell &
Sande, 1990). The structures of sulfanilamide,
sulfadiazine, sulfamethoxazole, sulfisoxazole and
sulfacetamide are shown below:

- 132 -
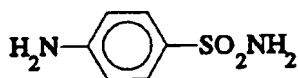
Sulfanilamide
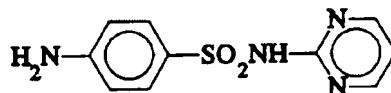
Sulfadiazine
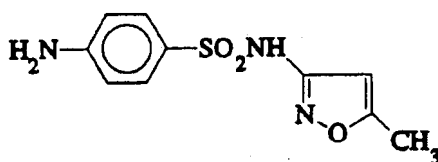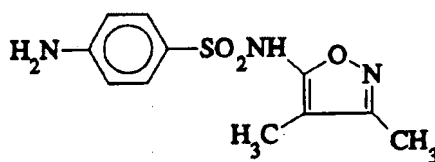
Sulfamethoxazole
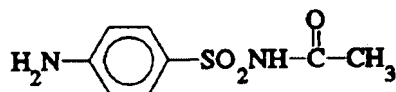
Sulfisoxazole

Sulfacetamide
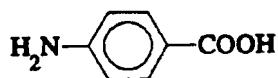
Para-aminobenzoic Acid

Clinical doses typically range from 2 to 8 g/day for sulfisoxazole as a typical example. Trimethoprim, a DHFR inhibitor is generally used in a combination with

5   sulfamethoxazole, a dihydropteroate synthetase inhibitor. This combination is important clinically for a number of microorganisms and is used at dosages of about 800/160 mg/day (SMX/TMP; Berkow et al., 1992).

10   Sulfadiazine (0.056 M) was evaluated by the inventors using the first described disc susceptibility procedure and was shown to enhance the relative activity of erythromycin against *S. aureus* 27660 by 183% (Table 8).

15

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EXAMPLE X
Inhibitors of Polyamine Synthesis

Igarashi et. al. (1979) showed a decrease in
polypeptide synthesis when an *E. coli* polyamine-requiring
mutant was grown in the absence of polyamines. This
suggested to the inventors that when inhibitors of
polyamine synthesis are combined with antimicrobials,
increased antimicrobial activity should be observed, due
to the reduction in methylase synthesis. A number of
agents capable of inhibiting polyamine synthesis are
described below.

α-methylornithine

Mach *et al.* (1982) reported that polyamines are
essential in prokaryotes and eukaryotes for optimal
growth and development. Polyamines had previously been
reported to bind in a specific way to both tRNAs and
ribosomes. Mach *et al.* (1982) presented data to show
that SAM-dependent transmethylation reactions can be
regulated by alterations of polyamine concentrations *in
vivo.* Methyltransferases from *Dictyostelium discoideum*
were almost inactive without the addition of added
polyamines. Additionally, for example, it was found that
the growth of *D. discoideum* was arrested in four hours
with 5 mM α-methylornithine.

α-methylornithine is available from Sigma Chemical
Company, and is proposed for use in combination with
antibiotics at doses of between about 1 and about 1000
mg/kg body weight/day, and preferably, between about 10
and about 200 mg/kg body weight/day.

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1,3-diaminopropan-2-ol

In the studies by Mach *et al.* (1982), the growth of
D. discoideum was also arrested in four hours using 5 mM
1,3-diaminopropan-2-ol. This compound is commercially
available from Fluka (Buchs, Switzerland), and is also
proposed for use at doses of between about 1 and about
1000 mg/kg body weight/day, and preferably, between about
10 and about 200 mg/kg body weight/day.

Difluoromethylornithine and Difluoromethylarginine

Tyms *et al.* (1988) reviewed the use of polyamine
inhibitors in antimicrobial chemotherapy. Concerning
difluoromethylornithine (DFMO), an irreversible inhibitor
of ornithine decarboxylase (ODC), they indicated that
some bacteria may not show growth inhibition since an
alternative biosynthetic pathway for putrescine exists in
these organisms.

To block putrescine biosynthesis in some procaryotic
cells using DMFO, inhibition of an additional pathway is
necessary. This can be achieved by the prevention of
arginine formation through inhibition of arginine
decarboxylase activity, e.g., using
difluoromethylarginine (DFMA), another catalytically
activated, irreversible inhibitor.

It has been shown in clinical trials that DFMO is
effective against *Trypanosomiasis* at different dosage
levels and modes of treatment by different groups. As an
example, DFMO was administered in one study at 400 mg/kg
qid, for 4 to 6 weeks. In another study, DFMO was
administered 20g i.v. for 11 days, 30 g daily for 2 days
and 15g orally every day for 7 weeks. Tyms (1988)
concluded that DFMO is safe and well tolerated and the

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side effects are reversible. Additionally, DL-α-
monofluoromethyldehydroornithine methylester, a DFMO
analogue, is taken up more readily than DFMO (K$_i$=39 μM)
and readily cleaved to the free amino acid within the
5   cell and has a K$_i$ of 3 μM.

The present inventors therefore propose that DFMO,
DFMA or a combination of DFMO and DFMA, will be effective
as inhibitory agents, or as an inhibitory package, for
10   use with an antimicrobial agent, such as an MLS
antibiotic. It is proposed that DFMO or analogues alone
or in combination with DFMA could be used successfully
with antimicrobials to kill microorganisms at doses of
about 1 to 10,000 mg/kg per day, and preferably 50 to
15   1000 mg/kg/day. DFMO (0.046 M) was evaluated by the
inventors using the first disc susceptibility procedure
and shown to enhance the relative activity of
erythromycin against *S. aureus* by 250% (Table 8).

20   **Inhibitors of S-Adenosylmethionine Decarboxylase (SAM-DC)**

The potential importance of inhibitors of the first
enzyme in the synthesis of polyamines have been
demonstrated by the success of DFMO, an inhibitor of
25   ornithine decarboxylase, in the treatment of
Trypanosomiasis. In order to convert putrescine into
polyamines spermine and spermidine, the aminopropyl group
is supplied by the catalytic activity of SAM-DC.
Therefore, the action of SAM decarboxylase is essential
30   for the synthesis of polyamines. It is therefore
expected that inhibitors of SAM decarboxylase will
operate in a similar fashion to ornithine decarboxylase
inhibitors.

35   Pegg & McCann (1992) suggested that SAM-DC
inhibitors may be of therapeutic value, either alone or

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in combination with ornithine decarboxylation inhibitors.
These authors described powerful irreversible inhibitors
of SAM-DC that are available, including
5'-([(Z)-4-amino-2-butenyl]methylamino)-5'-

5   deoxyadenosine, an enzyme activated inhibitor, and
5'-deoxy-5'-[(3-hydrazinopropyl)methylamino]adenosine,
which binds to the active site and forms a covalent bond
with the pyruvate prosthetic group. The teachings of
Pegg & McCann (1992) regarding SAM-DC inhibitors in the

10   treatment of tumors and, particularly, in the treatment
of protozoan parasites, is relevant to the choice of
SAM-DC inhibitors and appropriate doses for use in the
combined antimicrobial therapy of the present invention.

15   A number of useful inhibitors of SAM-DC have been
developed based on substrate or product analogues or
compounds that form a covalent bond with the pyruvate co-
factor. Some of the important inhibitors are AMA (S-(5'-
deoxy-5'-adenosyl)methylthioethylhydroxylamine), MHZPA

20   (5'-deoxy-5'-[(3-hydrazinopropyl)methylamino]adenosine),
MAOEA (5'-deoxy-5'-[(2-aminooxyethyl)-
methylamino]adenosine, AbeAdo (5'-{[(Z)-4-amino-2-
butenyl]methylamino}-5'-deoxyadenosine [MDL 73811]),
AdoMac (S-(5'-deoxy-5'-adenosyl)-1-amino-4-methylthio-2-

25   cyclopentene, MGBG (methylglyoxal bis(guanyl)hydrazone),
CGP-39'937 and CGP-33'829.

          Pegg & McCann (1992), reported that AbeAdo is at
least 100-fold more potent than DFMO, which is now an

30   approved drug for West African sleeping sickness. It is
proposed that AbeAdo and analogues alone, or in
combination with DFMA and or DFMO, could be used
successfully with antibiotics to kill bacteria. The
proposed dosages of AbeAdo and analogues is from about 1

35   to about 1,000 mg/kg per day, and preferably, from about
1 to about 200 mg/kg/day.
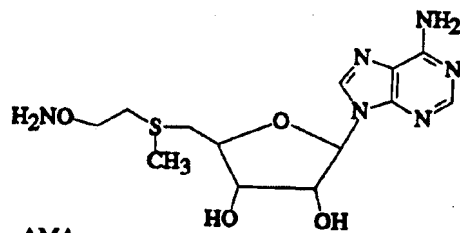
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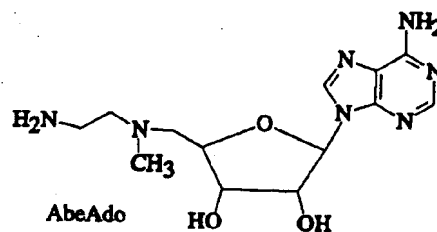
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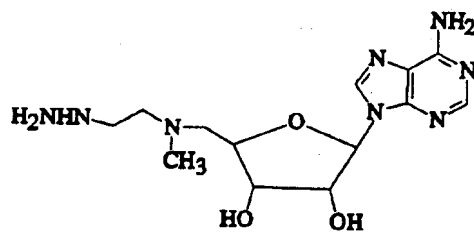
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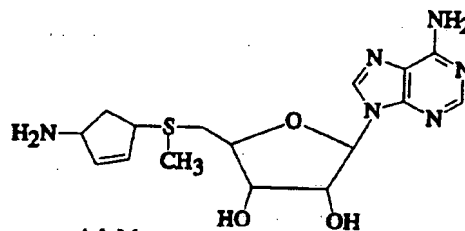
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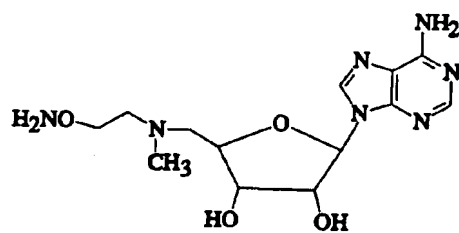
MAOEA

- 138 -
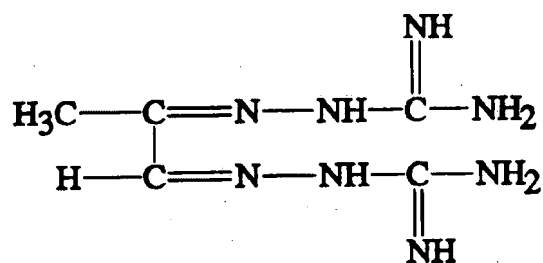
MGBG
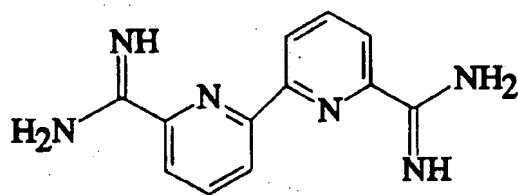
CGP-39'937
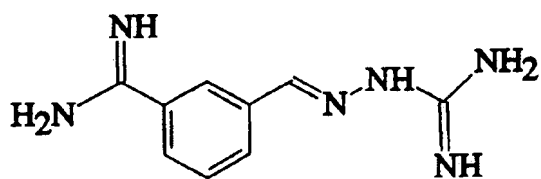
CGP-33'829

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Inhibitors of Ornithine Decarboxylase (ODC)

McCann & Pegg (1992) reported that ornithine
decarboxylase (ODC) is a suitable enzyme target for use
in certain therapeutic regimens. These authors describe
the therapeutic effects resulting from ODC inhibition and
particularly describe the inhibitor, eflornithine.
Recent in vivo therapeutic approaches to ODC inhibition
are also described, as may be applied cancer treatment,
cancer chemoprevention, autoimmune diseases, ischemia,
hyperplasia, hearing loss, African trypanosomiasis, AIDS,
and other infectious diseases/organisms. In light of the
teachings of McCann & Pegg (1992), and the present
disclosure, the use of ODC inhibitors can be readily
adapted for combination therapy with antimicrobials in
the treatment of microbial infections and diseases.

Inhibitors of Arginine Decarboxylase (ADC)

As stated above, Tyms et al. (1988) reported on the
inhibition of polyamine synthesis by preventing arginine
formation. This is achieved through the inhibition of
arginine decarboxylase (ADC). DFMA is a irreversible
inhibitor of ADC, which is contemplated for use in the
present invention at similar doses to those quoted above
for DFMO. Kallio et. al. (1981) described
DL-α-(difluoromethyl)arginine as a potent and
irreversible inhibitor of bacterial ADC.
DL-α-(difluoromethyl)arginine (RMI 71669) will thus be
effective alone and also in combination with ODC
inhibitors. In the latter case, all putrescine
biosynthesis may be inhibited in prokaryotic cells.

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EXAMPLE XI
Inhibitors of RNA Maturation

5-Azacitidine

5   5-Azacitidine inhibits the maturation of rRNA
(Suhudolnik, 1979). The formation of 28S and 18S, but
not of 38S RNA, is severely inhibited. 5-Azacitidine is
commercially available from Sigma Chemical Company. The
10   structure of 5-Azacitidine is shown below. Budavari
et al. (1989) reported the oral toxicity of 5-azacitidine
as 572.3 mg/kg in mice. This compound is therefore
proposed for use with antibiotics at doses of between
about 1 and about 300 mg/kg body weight/day, and
15   preferably, between about 10 and about 75 mg/kg body
weight/day.

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25

30
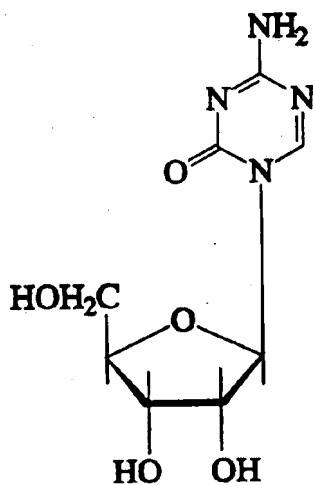
Cordycepin (3'-deoxyadenosine)

35   Cordycepin (Suhudolnik, 1979) inhibits the synthesis
of mature rRNA, ribosomal precursor 45S RNA and tRNA in

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Hela cells; DNA and protein synthesis are not affected. Cordycepin at 200 μg/ml totally inhibits RNA synthesis and at lower concentrations, rRNA and tRNA syntheses are selectively inhibited, but protein synthesis is not

5   effected. Cordycepin is commercially available from Sigma Chemical Company and it's structure is shown below. It is proposed for use with antibiotics at doses of between about 1 and about 250 mg/kg body weight/day, and preferably, between about 5 and about 75 mg/kg body

10   weight/day.

Toyocamycin and Analogues

Suhadolnik (1979) indicated that tubercidin,

15   toyocamycin and 6-thioguanosine inhibit the formation of mature 28S and 18S rRNA in Novikoff hepatoma cells. Similarly, toyocamycin was reported to inhibit the maturation of rRNA in *S. cerevisiae*, and is proposed for use with, e.g., MLS antibiotics, at doses of between

20   about 0.1 and about 100 mg/kg body weight/day, and preferably, between about 0.1 and about 50 mg/kg body weight/day.

                          EXAMPLE XII

25   <u>ANTISENSE OLIGONUCLEOTIDES</u>

Cohen & Hogan (1994; incorporated herein by reference) describe the general approach used to develop synthetic oligonucleotides as drugs.

30

Translation Inhibition

DNA or RNA antisense oligonucleotides are also contemplated as another means of inhibiting bacterial

35   methylation. In such methods, oligonucleotides capable of hybridizing with those portions of the mRNA sequences

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that are involved in binding to the ribosome or to the
methylation site of the rRNA may be employed. This will
block the induction of the bacterial methylase synthetase
and thus reduce antibiotic resistance when used in
5   combination with an antibiotic. Exemplary sequences are
set forth below, modifications of which may be readily
designed using the teaching herein and the known
sequences and structural information, such as that
described by Dubnau (1984; 1985) and Denoya et al.
10   (1986).

ermC is known to specify an rRNA methyltransferase
that confers resistance to erythromycin. The transferase
N^6,N^6-dimethylates an adenine residue at position 2058 on
15   the 23S rRNA, thereby decreasing ribosomal affinity for
erythromycin. Basically the ermC mRNA exists in an
inactive conformation in which the ribosome binding site
for methylase synthesis, the Shine Delgarno sequence 2
(SD2), is sequestered by base pairing. Stalling of a
20   ribosome under the influence of erythromycin binding
during translation of a 19-amino acid leader peptide
causes the inactive mRNA structure to open, and free the
sequestered SD2 permitting initiation of methylase
synthesis.
25

The structure of the ermC mRNA regulatory region is
shown in FIG. 1A and FIG. 1B of Dubnau (1985) and FIG. 3
of Dubnau (1984). In this region, the ribosomal binding
site for the putative leader peptide is defined as SD1.
30   SD1 has the sequence AGGAGGA, which is followed six
residues down by the initiation codon ATG (also referred
to as AUG). The complete SD1 site, AGGAGGAAAAATATG (SEQ
ID NO:1), is the primary target proposed for antisense
inhibition.
35

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The inventors have designed complementary
oligonucleotide sequences against this region that will
hybridize to the SD1 + initiation codon region, thus
preventing binding of this mRNA to the ribosome. A
5   candidate complementary, or antisense, oligonucleotide
is: TCCTCCTTTTTTATAC (SEQ ID NO:2), or UCCUCCUUUUUUAUAC
(the ribooligonucleotide, SEQ ID NO:3).

In the *ermC* mRNA region, there is also an additional
10   ribosomal binding site, SD2. This has a regulatory role
and is usually translationally inactive due to base
pairing. SD2 and the initiation codon has the sequence
AGAGGGTTATAATG (SEQ ID NO:4). Since the *ermC* mRNA
structure in the regulatory region can re-arrange into
15   different conformations, and thus open the SD2 sequence,
an oligonucleotide complementary to the SD2 + initiation
codon region is also proposed for use in arresting
translation of the methylase enzyme. An appropriate
candidate antisense deoxy oligonucleotide is:
20   TCTCCCAACACTAC (SEQ ID NO:5).

Oligonucleotides will be designed and synthesized
(Gait, 1984; Zon & Geiser, 1991). The appropriate length
of the oligonucleotide to allow efficient hybridization
25   at physiological salt concentration and temperature will
be determined by computer analysis (Rychlik & Rhoads,
1989; Van Ness & Chen, 1991). These oligonucleotide will
contain appropriate modifications that will allow
sufficient stability *in vivo* and allow adequate transport
30   into the cell. These oligonucleotide will be evaluated
in vitro by their ability to prevent synthesis of the
rRNA methylase. Active compound identified *in vitro*
in vivo will then be evaluated *in vivo* as a "new
antibiotic", as described by Lorian (1991).

35

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Decoy Substrates

Oligoribonucleotides with sufficient stability are
also contemplated for use as decoys to serve as
5   substrates for the methylase enzyme. The "decoy oligos"
would have tight binding properties and function to bind
with, and saturate, the active-site of the methylase
enzyme. Appropriate decoy sequences are those
surrounding the target site of dimethylation on the 23S
10   rRNA and the autoregulatory site on the *ermC* mRNA. The
oligos used may have the sequences as taught below or
variations thereof designed using this disclosure and the
sequences and structural information described in the
literature, e.g., FIG. 6 of Denoya *et al.* (1986;
15   incorporated herein by reference).

Particular inhibitory structures proposed by the
inventors are set forth below and are represented by SEQ
ID NO:6, SEQ ID NO:7 and SEQ ID NO:8. Binding to the
20   double stranded sequence is preferred. The sequence will
depend on the organisms targeted and may contain
degenerated sequences. These structures can also contain
any group or modification that the enzyme will tolerate.

25   A   C
                    GCGAC GGA GGAAAGACCCC
                    CGCUG CCU CC   GGGG
                             AU

30   and

                         A   C
                    GCGAC GGA GGAAAGACCCC

35   Additionally, the potency of the decoy oligos can be
improved by designing a group into the molecule that has
the ability to covalently attach to the enzyme and
permanently inactivate it. Many different groups that
function in this capacity are known in the art. For

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example, the reactive oligonucleotide derivatives used as
gene-targeted biological active compounds, that are
currently in use in this field, have recently been
reviewed Knorre & Vlassov (1991). These authors
5 indicated that since it appears that binding of
deoxyribonucleotides to the coding region of mRNAs does
not effect translation, because the translating ribosome
displaces bound oligonucleotides, coupling of reactive
groups that crosslink to form covalent bonds with the
10 target will create an insurmountable block for the
ribosome. Examples of modifications coupled to
oligonucleotides that crosslink to target are 2-
chlorethylamino-, EDTA Fe- and o-phenanthroline Cu-
groups. Additionally groups that increase binding to
15 nucleic acid targets could also be used, such as
phenazinium-conjugated oligonucleotides.

Specific Bacterial Targets

20 It will be understood that, generally, the sequence
of methylase mRNAs and rRNAs from different bacteria will
not be identical. The sequence variations will need to
be considered when designing oligonucleotides for use in
connection with specific bacteria. Sequences are readily
25 available from data bases, such as GenBank.

Modifications

  Depending on the Tm' of the oligonucleotide:mRNA
30 hybrid, the length of the oligonucleotide will be
optimized for maximum activity.

  In addition to any of the common bases, the
oligonucleotides may also contain other naturally
35 occurring or modified bases. For example, both
deoxyribonucleotides and ribonucleotides may contain

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backbone modifications, including methylphosphonates,
phosphorothioates, phosphorodithioates and PNAs (protein
nucleic acids; sugar modifications, including 2'-alkyl
groups; base modifications, including inosine,
5   alphanucleoside, and 3' and 5' blocking groups, including
cholesterol, 3' and 5' alkylhydroxyl groups, and
interchelators.

Such modifications, individually or in combination,
10 are proposed for use in designing an oligonucleotide that
hybridizes specifically to the initiation target and
additionally contains sufficient stability against
nuclease degradation. Certain preferred oligonucleotides
include 3'- and/or 5'-blocked oligodeoxynucleic acids.
15 The advantage of such oligodeoxynucleotides is that the
endogenous RNase H, would degrade the complementary mRNA
when the oligodeoxynucleotide/mRNA hybrid. This is
contemplated to allow permanent inhibition of the
translation of this particular methylase mRNA.
20

Oligonucleotide Delivery

A technology that allows the transport of
oligonucleotides into bacteria has recently been
25 developed (Couvreur et al., 1991; incorporated herein by
reference) and is commercially available from Genta
Corporation, San Diego, CA.

EXAMPLE XIII
30 **PHARMACEUTICAL COMPOSITIONS**

Compositions of the present invention may comprise a
combined effective amount of an antimicrobial agent or
antibiotic and an agent that inhibits rRNA methylation or
35 maturation, or such agents may be provided as distinct
compositions, intended for use in combination. The

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antibiotics, second agent inhibitors or combinations
thereof, may be dissolved or dispersed in a
pharmaceutically acceptable carrier or aqueous medium, or
may be provided in solid form.

5

The phrases "pharmaceutically or pharmacologically
acceptable" refer to molecular entities and compositions
that do not produce adverse, allergic or other untoward
reactions when administered to an animal, or a human, as
10   appropriate. As used herein, "pharmaceutically
acceptable carrier" includes any and all solvents,
dispersion media, coatings, additional antibacterial and
antifungal agents, isotonic and absorption delaying
agents, and the like (USP XXII, The United States
15   Pharmacopeia, 1990; incorporated herein by reference).

The use of such media and agents for pharmaceutical
active substances is well known in the art. Except
insofar as any conventional media or agent is
20   incompatible with the active ingredient, its use in the
therapeutic compositions is contemplated. Supplementary
active ingredients, particularly other non-MLS
antibiotics, can also be incorporated into the
compositions.

25

In addition to the detailed information provided
herein, the teachings of Eliopoulos & Moellering (1991)
and Shungu (1991) are incorporated by reference for the
purposes of further describing appropriate antimicrobial
30   combinations and the use of antibiotics in medicine.

Kits for use in the present invention are
contemplated to include various combinations of
solutions, tablets, ointments and other formulations, and
35   also to encompass two component systems. One component
will be at least one inhibitor that will be administered,

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generally at a predetermined time before the MLS
antibiotic (the second component). Additional components
may be used in the context of the therapeutic methods
provided by this invention, although they may not be
5   included in a kit per se. For example, N$_2$O, used
extensively in dentistry, may be employed in essentially
in the same way, and given simultaneously and/or as a
pre-treatment to antibiotic therapy.

10   **Parenteral Formulations**

The active compounds, whether antibiotics, second
agent inhibitors, or combinations thereof, may be
formulated for parenteral administration, e.g.,
15   formulated for injection via the intravenous,
intramuscular, sub-cutaneous, or even intraperitoneal
routes (USP XXII, The United States Pharmacopeia, 1990).
The preparation of an aqueous composition that contains
such active ingredients, or combinations thereof, will be
20   known to those of skill in the art in light of the
present disclosure. Typically, such compositions can be
prepared as injectables, either as liquid solutions or
suspensions. Solid forms suitable for using to prepare
solutions or suspensions upon the addition of a liquid
25   prior to injection can also be prepared, and the
preparations can also be emulsified.

Solutions of each or both of the active compounds as
free base or pharmacologically acceptable salts can be
30   prepared in water suitably mixed with a surfactant (USP
XXII, The United States Pharmacopeia, 1990) such as
hydroxypropylcellulose. Dispersions can also be prepared
in glycerol, liquid polyethylene glycols, in mixtures
thereof and in oils. Under ordinary conditions of
35   storage and use, these preparations will contain a
preservative to prevent the growth of microorganisms.

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The pharmaceutical forms suitable for injectable use
include: aqueous solutions or dispersions, formulations
including sesame oil, peanut oil or aqueous propylene
glycol and powders for the extemporaneous preparation of
5   injectable solutions or dispersions. In all cases the
form must be sterile and must be fluid to the extent that
easy syringability exists. It must be stable under the
conditions of manufacture and storage and must be
preserved against the contaminating action of
10   microorganisms, such as bacteria and fungi.

The antibiotics and second inhibitory agents can be
formulated into one or more compositions in a neutral or
salt form. Pharmaceutically acceptable salts, include
15   the acid addition salts (formed with the free amino
groups of the protein) which are formed with inorganic
acids such as, for example, hydrochloric or phosphoric
acids, or such organic acids as acetic, oxalic, tartaric,
mandelic, lactic and the like. Salts formed with the
20   free carboxyl groups can also be derived from inorganic
bases such as, for example, sodium, potassium, ammonium,
calcium, or ferric hydroxides, and such organic bases as
isopropylamine, trimethylamine, histidine, procaine and
the like.
25

The carrier can also be a solvent or dispersion
medium containing, for example, water, ethanol, polyol
(for example, glycerol, propylene glycol, and liquid
polyethylene glycol, and the like), suitable mixtures
30   thereof, and vegetable oils. The proper fluidity can be
maintained, for example, by the use of a coating such as
lecithin, by the maintenance of the required particle
size in the case of dispersion and by the use of
surfactants. The prevention of the action of
35   microorganisms can be brought about by various
antibacterial and antifungal agents, for example,

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parabens, chlorobutanol, phenol, sorbic acid, thimerosal,
and the like. In many cases, it will be preferable to
include isotonic agents, for example, sugars or sodium
chloride. Prolonged absorption of the injectable
5   compositions can be brought about by the use in the
compositions of agents delaying absorption, for example,
aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by
10   incorporating the active compounds in the required amount
in the appropriate solvent with various of the other
ingredients enumerated above, as required, followed by
filter sterilization. Generally, dispersions are
prepared by incorporating the various sterilized active
15   ingredients into a sterile vehicle which contains the
basic dispersion medium and the required other
ingredients from those enumerated above. In the case of
sterile powders for the preparation of injectable
solutions, the preferred methods of preparation are
20   vacuum-drying and freeze-drying techniques which yield a
powder of the active ingredient plus any additional
desired ingredient from a previously sterile-filtered
solution thereof.

25   The preparation of more, or highly, concentrated
solutions for intramuscular injection is also
contemplated. This is envisioned to have particular
utility in cases where either the antibiotic or the
inhibitor is sensitive to digestive conditions. In this
30   regard, the use of DMSO as solvent is preferred as this
will result in extremely rapid penetration, delivering
high concentrations of the active agents to a small area.

Upon formulation, solutions will be administered in
35   a manner compatible with the dosage formulation and in
such amount as are therapeutically effective, as outlined

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in the foregoing examples. The formulations are easily
administered in a variety of dosage forms. For
parenteral administration in an aqueous solution, for
example, the solution should be suitably buffered if
5   necessary and the liquid diluent first rendered isotonic
with sufficient saline or glucose. These particular
aqueous solutions are especially suitable for
intravenous, intramuscular, subcutaneous and
intraperitoneal administration. In this connection,
10   sterile aqueous media which can be employed will be known
to those of skill in the art in light of the present
disclosure. For example, one dosage could be dissolved
in 1 ml of isotonic NaCl solution and either added to
1000 ml of hypodermoclysis fluid or injected at the
15   proposed site of infusion, (see for example, "Remington's
Pharmaceutical Sciences" 15th Edition, pages 1035-1038
and 1570-1580). Some variation in dosage will
necessarily occur depending on the condition of the
subject being treated. The person responsible for
20   administration will, in any event, determine the
appropriate dose for the individual subject.

    In addition to the compounds formulated for
parenteral administration, other pharmaceutically
25   acceptable forms are also contemplated for use,
including, e.g., tablets or other solids for oral
administration; time release capsules; forms for topical
administration, including creams and lotions;
mouthwashes; aerosols, inhalents and the like.
30

    The components for use with the invention may be
prepared in the form a treatment kit wherein the
antibiotic and inhibitory agent are located within a
suitable container, such as at least one vial, test tube,
35   flask, bottle, syringe or other container, into which the
active components are placed, and preferably, suitably

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allocated. Where desired, the antibiotic and inhibitor
may be provided in distinct containers, such that the kit
will contain two vials, or other containers, each of
which contains only one of the active agents. These
5   "therapeutic kits" will also typically include a means
for containing the vials or the containers in close
confinement for commercial sale, such as, e.g., injection
or blow-molded plastic containers into which the desired
vials are retained.

10

Oral Formulations

In certain embodiments, active compounds may be
administered orally. This is contemplated for agents
15   that are generally resistant, or have been rendered
generally resistant, to proteolysis by digestive enzymes.
Such compounds are contemplated to include the
antibiotics and most of the inhibitory second agents
disclosed herein. Naturally, the preferred inhibitors
20   will be the more active compounds and those already
cleared by the FDA for other uses, as will be known to
those of skill in the art in light of the present
disclosure.

25   For oral administration, the active compounds and/or
combinations thereof may be administered, for example,
with an inert diluent or with an assimilable edible
carrier, or they may be enclosed in hard or soft shell
gelatin capsule, or compressed into tablets, or
30   incorporated directly with the food of the diet. For
oral therapeutic administration, the active compounds may
be incorporated with excipients and used in the form of
ingestible tablets, buccal tables, troches, capsules,
elixirs, suspensions, syrups, wafers, and the like. Such
35   compositions and preparations should contain at least
0.1% of active compounds or combinations thereof. The

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percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 2 to about 60% of the weight of the unit. The amount of active compounds in such therapeutically useful

5   compositions is such that a suitable dosage will be obtained.

        The tablets, troches, pills, capsules and the like may also contain the following: a binder, as gum

10 tragacanth, acacia, cornstarch, or gelatin; excipients, such as dicalcium phosphate; a disintegrating agent, such as corn starch, potato starch, alginic acid and the like; a lubricant, such as magnesium stearate; a sweetening agent, such as sucrose, lactose or saccharin may be added

15 or a flavoring agent, such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings to modify the

20 physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar or both. A syrup of elixir may contain the active compounds sucrose as a sweetening agent methyl and propylparabens as preservatives, a dye and flavoring, such as cherry or

25 orange flavor. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compounds may be incorporated into sustained-release preparation and formulations.

30

Topical Formulations

        The formulation of antimicrobial agents, such as antibiotics, and second, inhibitory agents for topical

35 use, such as in creams, ointments and gels is also contemplated. The preparation of oleaginous or water-

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soluble ointment bases is also well known to those in the
art. For example, these compositions may include
vegetable oils, animal fats, and more preferably,
semisolid hydrocarbons obtained from petroleum.
5   Particular components used may include white ointment,
yellow ointment, cetyl esters wax, oleic acid, olive oil,
paraffin, petrolatum, white petrolatum, spermaceti,
starch glycerite, white wax, yellow wax, lanolin,
anhydrous lanolin and glyceryl monostearate. Various
10   water-soluble ointment bases may also be used, including
glycol ethers and derivatives, polyethylene glycols,
polyoxyl 40 stearate and polysorbates. Even delivery
through the skin may be employed if desired, e.g., by
using transdermal patches, ionophoresis or
15   electrotransport. Examples of hydrophilic and
hydrophobic ointments are known in the art, (e.g., USP
XXII, The United States Pharmacopeia, 1990).

Nasal Solutions and Inhalants

20

     One may also use nasal solutions or sprays, aerosols
or inhalants with the antimicrobials and second agents
described herein. Nasal solutions are usually aqueous
solutions designed to be administered to the nasal
25   passages in drops or sprays. Nasal solutions are
prepared so that they are similar in many respects to
nasal secretions, so that normal ciliary action is
maintained. Thus, the aqueous nasal solutions usually
are isotonic and slightly buffered to maintain a pH of
30   5.5 to 6.5. In addition, antimicrobial preservatives,
similar to those used in ophthalmic preparations, and
appropriate drug stabilizers, if required, may be
included in the formulation. Various commercial nasal
preparations are known and include, for example, those
35   for use with various antibiotics and antihistamines.

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Inhalations and inhalants are pharmaceutical preparations designed for delivering a drug or compound into the respiratory tree of a patient. A vapor or mist is administered and reaches the affected area to give
5   relief from symptoms of bronchial and nasal congestion. Inhalations may be administered by the nasal or oral respiratory routes. The administration of inhalation solutions is only effective if the droplets are sufficiently fine and uniform in size so that the mist
10   reaches the bronchioles.

Another group of products, also known as inhalations, and sometimes called insufflations, consists of finely powdered or liquid drugs that are carried into
15   the respiratory passages by the use of special delivery systems, such as pharmaceutical aerosols, that hold a solution or suspension of the drug in a liquefied gas propellant. When released through a suitable valve and oral adapter, a metered does of the inhalation is
20   propelled into the respiratory tract of the patient.

Particle size is of major importance in the administration of this type of preparation. It has been reported that the optimum particle size for penetration
25   into the pulmonary cavity is of the order of 0.5 to 7 μm. Fine mists are produced by pressurized aerosols and hence their use in considered advantageous. The active compounds described above, whether antibiotics, second agent inhibitors, or combinations thereof, may be
30   formulated for nasal administration in any of these methods.

Ophthalmic Solutions

35   The antimicrobials and agents that inhibit rRNA methylation or maturation may also be used in

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preparations of pharmaceutical compositions suitable for
use as ophthalmic solutions. The preparation of
ophthalmic formulations in accordance with conventional
pharmaceutical practice is well known, see for example
5   "Remington's Pharmaceutical Sciences" 15th Edition, pages
1488 to 1501 (Mack Publishing Co., Easton, PA).

The ophthalmic preparations may contain antibiotics
and/or second agents, or pharmaceutically acceptable
10   salts thereof, in a concentration from about 0.01 to
about 1% by weight, preferably from about 0.05 to about
0.5% in a pharmaceutically acceptable solution,
suspension or ointment. Some variation in concentration
will necessarily occur, depending on the particular
15   compound employed, the condition of the subject to be
treated and the like, and the person responsible for
treatment will determine the most suitable concentration
for the individual subject. The ophthalmic preparation
will preferably be in the form of a sterile aqueous
20   solution containing, if desired, additional ingredients,
for example preservatives, buffers, tonicity agents,
antioxidants and stabilizers, nonionic wetting or
clarifying agents, viscosity-increasing agents and the
like.
25

Suitable preservatives for use in such a solution
include benzalkonium chloride, benzethonium chloride,
chlorobutanol, thimerosal and the like. Suitable buffers
include boric acid, sodium and potassium bicarbonate,
30   sodium and potassium borates, sodium and potassium
carbonate, sodium acetate, sodium biphosphate and the
like, in amounts sufficient to maintain the pH between
about 6 and 8, and preferably, between about pH 7 and pH
7.5. Suitable tonicity agents are dextran 40, dextran
35   70, dextrose, glycerin, potassium chloride, propylene
glycol, sodium chloride, and the like, such that the

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sodium chloride equivalent of the ophthalmic solution is
in the range 0.9 plus or minus 0.2%. Suitable
antioxidants and stabilizers include sodium bisulfite,
sodium metabisulfite, sodium thiosulfate, thiourea and
5   the like. Suitable wetting and clarifying agents include
polysorbate 80, polysorbate 20, poloxamer 282 and
tyloxapol. Suitable viscosity-increasing agents include
dextran 40, dextran 70, gelatin, glycerin,
hydroxyethylcellulose, hydroxymethylpropylcellulose,
10   lanolin, methylcellulose, petrolatum, polyethylene
glycol, polyvinyl alcohol, polyvinylpyrrolidone,
carboxymethylcellulose and the like. The ophthalmic
preparation will be administered topically to the eye of
the subject in need of treatment by conventional methods,
15   for example in the form of drops or by bathing the eye in
the ophthalmic solution.

Liposomes and Nanoparticles

20   Couvreur *et al.* (1991) review the potential of
liposomes and nanoparticles in the targeted antibiotic
therapy of intracellular bacterial infections and
diseases. Nanoparticles can generally entrap antibiotics
in a stable and reproducible way (Henry-Michelland
25   *et al.*, 1987). To avoid side effects due to
intracellular polymeric overloading, such ultrafine
particles (sized around 0.1 μm) should be designed using
polymers able to be degraded *in vivo*. Biodegradable
polyalkyl-cyanoacrylate nanoparticles that meet these
30   requirements are proposed for use in the present
invention. They are easily made, as described by
Couvreur *et al.* (1984; 1988). In serum, the liberation
of ampicillin from nanoparticles was found to follow
zero-order kinetics (Fattal *et al.*, 1991a, 1991b).

35

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    Liposomes are formed from phospholipids that are
dispersed in an aqueous medium and spontaneously form
multilamellar concentric bilayer vesicles (also termed
multilamellar vesicles (MLVs). MLVs generally have
5   diameters of from 25 nm to 4 μm. Sonication of MLVs
results in the formation of small unilamellar vesicles
(SUVs) with diameters in the range of 200 to 500 Å,
containing an aqueous solution in the core.

10   Liposomes bear many resemblances to cellular
membranes and are contemplated for use in connection with
the present invention as carriers for the antibiotics
and/or second agents. They are widely suitable as both
water- and lipid-soluble substances can be entrapped,
15   i.e., in the aqueous spaces and within the bilayer
itself, respectively. It is possible that drug-bearing
liposomes may even be employed for site-specific delivery
of active agents by selectively modifying the liposomal
formulation.
20

    The formation and use of liposomes is generally
known to those of skill in the art. For example,
Couvreur et al. (1991; incorporated herein by reference)
describe the use of liposomes and nanoparticles in the
25   targeted antibiotic therapy of intracellular bacterial
infections and diseases. Recently, liposomes were
developed with improved serum stability and circulation
half-times (Gabizon & Papahadjopoulos, 1988; Allen &
Choun, 1987).
30

    In addition to the teachings of Couvreur et al.
(1991), the following information may be utilized in
generating liposomal formulations. Phospholipids can
form a variety of structures other than liposomes when
35   dispersed in water, depending on the molar ratio of lipid
to water. At low ratios the liposome is the preferred

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structure. The physical characteristics of liposomes
depend on pH, ionic strength and the presence of divalent
cations. Liposomes can show low permeability to ionic
and polar substances, but at elevated temperatures
5   undergo a phase transition which markedly alters their
permeability. The phase transition involves a change
from a closely packed, ordered structure, known as the
gel state, to a loosely packed, less-ordered structure,
known as the fluid state. This occurs at a
10   characteristic phase-transition temperature and results
in an increase in permeability to ions, sugars and drugs.

In addition to temperature, exposure to proteins can
alter the permeability of liposomes. Certain soluble
15   proteins such as cytochrome c bind, deform and penetrate
the bilayer, thereby causing changes in permeability.
Cholesterol inhibits this penetration of proteins,
apparently by packing the phospholipids more tightly. It
is contemplated that the most useful liposome formations
20   for antibiotic and inhibitor delivery will contain
cholesterol.

The ability to trap solutes varies between different
types of liposomes. For example, MLVs are moderately
25   efficient at trapping solutes, but SUVs are extremely
inefficient. SUVs offer the advantage of homogeneity and
reproducibility in size distribution, however, and a
compromise between size and trapping efficiency is
offered by large unilamellar vesicles (LUVs). These are
30   prepared by ether evaporation and are three to four times
more efficient at solute entrapment than MLVs.

In addition to liposome characteristics, an
important determinant in entrapping compounds is the
35   physicochemical properties of the compound itself. Polar
compounds are trapped in the aqueous spaces and nonpolar

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compounds bind to the lipid bilayer of the vesicle.
Polar compounds are released through permeation or when
the bilayer is broken, but nonpolar compounds remain
affiliated with the bilayer unless it is disrupted by
5   temperature or exposure to lipoproteins. Both types show
maximum efflux rates at the phase transition temperature.

Liposomes interact with cells via four different
mechanisms: Endocytosis by phagocytic cells of the
10   reticuloendothelial system such as macrophages and
neutrophils; adsorption to the cell surface, either by
nonspecific weak hydrophobic or electrostatic forces, or
by specific interactions with cell-surface components;
fusion with the plasma cell membrane by insertion of the
15   lipid bilayer of the liposome into the plasma membrane,
with simultaneous release of liposomal contents into the
cytoplasm; and by transfer of liposomal lipids to
cellular or subcellular membranes, or vice versa, without
any association of the liposome contents. It often is
20   difficult to determine which mechanism is operative and
more than one may operate at the same time.

The fate and disposition of intravenously injected
liposomes depend on their physical properties, such as
25   size, fluidity and surface charge. They may persist in
tissues for hours or days, depending on their
composition, and half lives in the blood range from
minutes to several hours. Larger liposomes, such as MLVs
and LUVs, are taken up rapidly by phagocytic cells of the
30   reticuloendothelial system, but physiology of the
circulatory system restrains the exit of such large
species at most sites. They can exit only in places
where large openings or pores exist in the capillary
endothelium, such as the sinusoids of the liver or
35   spleen. Thus, these organs are the predominate site of
uptake. On the other hand, SUVs show a broader tissue

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distribution but still are sequestered highly in the
liver and spleen. In general, this *in vivo* behavior
limits the potential targeting of liposomes to only those
organs and tissues accessible to their large size. These
5   include the blood, liver, spleen, bone marrow and
lymphoid organs.

Targeting is generally not a limitation in terms of
the present invention. However, should specific
10   targeting be desired, methods are available for this to
be accomplished. Antibodies may be used to bind to the
liposome surface and to direct the antibody and its drug
contents to specific antigenic receptors located on a
particular cell-type surface. Carbohydrate determinants
15   (glycoprotein or glycolipid cell-surface components that
play a role in cell-cell recognition, interaction and
adhesion) may also be used as recognition sites as they
have potential in directing liposomes to particular cell
types. Mostly, it is contemplated that intravenous
20   injection of liposomal preparations would be used, but
other routes of administration are also conceivable.

Using the combined antimicrobial formulations
described herein in liposomal formulations will lead to
25   further particular advantages that relate to targeting.
The properties of liposomes and nanoparticles will likely
improve therapy in a number of bacterial infections that
are presently difficult to cure. As set forth by
Couvreur *et al.* (1991), liposomes and nanoparticles
30   themselves cannot escape from the circulation because of
the endothelial barrier, with exception to tissues with
discontinuous endothelia lining their capillaries (i.e.,
liver, spleen, and bone marrow). This leads to the rapid
clearance of these ultrafine particulate carriers from
35   the blood and to their capture by the cells of the
reticuloendothelial system (Poste, 1983; Grislain *et al.*,

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1983). This corresponds exactly to the same tissue
distribution pattern as that of the majority of the
bacteria responsible for intracellular infection. In
addition, it has been shown that liposomes are taken up
5   by circulating blood monocytes, which are known to
infiltrate some infectious lesions (Tulkens, 1985).
Also, as with bacteria, both liposomes and nanoparticles
will likely penetrate cells by endocytosis, first forming
phagosomes, which in turn fuse with lysosomes to form
10   phagolysosomes or secondary lysosomes (Couvreur et al.,
1977).

The advantages of liposomes and nanoparticles has
been described for various cases. Liposome-associated
15   cephalothin has been shown to be more effective than free
drug in the treatment of experimental murine
salmonellosis (Desiderio and Campbell, 1983). The
therapeutic index of ampicillin, calculated on the basis
of mouse mortality, has been reported to be increased by
20   120-fold upon binding to nanoparticles (Fattal et al.,
1989). Guinea pigs infected by Brucella canis and
treated with liposome-entrapped streptomycin were found
to be free of bacteria (2 x 10 mg/kg), whereas animals
treated with the free drug, for the same schedule of
25   administration, showed only a minor reduction in the
number of surviving bacteria (Fountain et al., 1985).
The delivery of antibacterial drugs to phagocytic cells
has also been demonstrated to be feasible with amikacin
(Düzgünes et al., 1988), kanamycin and tobramycin
30   (Fountain et al., 1985).

Therapeutic Kits

Therapeutic kits comprising antimicrobial agents and
35   methylation inhibitors are contemplated and will
generally contain, in suitable container means, any of

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the previously described or other suitable
pharmaceutically acceptable formulations of one or more
antibiotics and inhibitors. The kit may have a single
container means for both (or all) components or it may
5   have more than one distinct container means.

When the components of the kit are provided in one
or more liquid solutions, the liquid solution will
generally be an aqueous solution, with a sterile aqueous
10   solution being particularly preferred. However, the
components may also be formulated into a syringable
composition. In which case, the container means may
itself be a syringe, pipette, eye dropper, or other such
like apparatus, from which the formulation may be applied
15   to an infected area of the body, injected into an animal,
or even applied to and mixed with the other components of
the kit.

The components of the kit may also be provided as
20   dried powder(s). When reagents or components are
provided as a dry powder, the powder can be reconstituted
by the addition of a suitable solvent. It is envisioned
that the solvent may also be provided in another
container means. The container means will generally
25   include at least one vial, test tube, flask, bottle,
syringe or other container means, into which the
antibiotics and methylation inhibitors may be placed,
preferably, suitably allocated. The kits may also
comprise the second/third container means mentioned above
30   for containing a sterile, pharmaceutically acceptable
buffer or other diluent.

The kits of the present invention will also
typically include a means for containing the vials in
35   close confinement for commercial sale, such as, e.g.,
injection or blow-molded plastic containers into which

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the desired vials are retained. Irrespective of the
number or type of containers, the kits of the invention
may also comprise, or be packaged with, an instrument for
assisting with the administration, placement or injection
5   of the components or combined composition within the body
of an animal. Such an instrument may be a syringe,
pipette, forceps, measured spoon, eye dropper or any such
medically approved delivery vehicle or device.

10

                            *   *   *

          All of the compositions and methods disclosed and
claimed herein can be made and executed without undue
15   experimentation in light of the present disclosure.
While the compositions and methods of this invention have
been described in terms of preferred embodiments, it will
be apparent to those of skill in the art that variations
may be applied to the composition, methods and in the
20   steps or in the sequence of steps of the method described
herein without departing from the concept, spirit and
scope of the invention. More specifically, it will be
apparent that certain agents which are both chemically
and physiologically related may be substituted for the
25   agents described herein while the same or similar results
would be achieved. All such similar substitutes and
modifications apparent to those skilled in the art are
deemed to be within the spirit, scope and concept of the
invention as defined by the appended claims.

30

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SEQUENCE LISTING

(1) GENERAL INFORMATION:
5

        (i) APPLICANT:
            (A) NAME: Oridigm Corporation
            (B) STREET: #6 Nickerson Street, Suite 201
            (C) CITY: Seattle
10   (D) STATE: Washington
            (E) COUNTRY: USA
            (F) POSTAL CODE (ZIP): 98109

        (ii) TITLE OF INVENTION: Combinations and Methods for
15   Reducing Antimicrobial
                                 Resistance

        (iii) NUMBER OF SEQUENCES: 8

20   (iv) COMPUTER READABLE FORM:
            (A) MEDIUM TYPE: Floppy disk
            (B) COMPUTER: IBM PC compatible
            (C) OPERATING SYSTEM: PC-DOS/MS-DOS
            (D) SOFTWARE: PatentIn Release #1.0, Version
25   #1.30 (EPO)

        (v) CURRENT APPLICATION DATA:
            (A) APPLICATION NUMBER: UNKNOWN
            (B) FILING DATE: CONCURRENTLY HEREWITH
30   (C) CLASSIFICATION: UNKNOWN

        (vi) PRIOR APPLICATION DATA:
            (A) APPLICATION NUMBER: US 08/364,246
            (B) FILING DATE: 27-DEC-1994
35

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(2) INFORMATION FOR SEQ ID NO: 1:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 15 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AGGAGGAAAA ATATG   15

(2) INFORMATION FOR SEQ ID NO: 2:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 16 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TCCTCCTTTT TTATAC   16

(2) INFORMATION FOR SEQ ID NO: 3:

    (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 16 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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UCCUCCUUUU UUAUAC   16

    (2) INFORMATION FOR SEQ ID NO: 4:
5

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 14 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
10   (D) TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AGAGGGTTAT AATG   14
15

    (2) INFORMATION FOR SEQ ID NO: 5:

        (i) SEQUENCE CHARACTERISTICS:
20   (A) LENGTH: 14 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

25   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

    TCTCCCAACA CTAC   14

30 (2) INFORMATION FOR SEQ ID NO: 6:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 21 base pairs
            (B) TYPE: nucleic acid
35   (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

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(ix) FEATURE:
        (A) NAME/KEY: modified_base
        (B) LOCATION:one-of(6, 10)
        (D) OTHER INFORMATION:/mod_base= OTHER
                /note= "N = A, G, C, or T"


(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:


GCGACNGGAN GGAAAGACCC C                                          21



(2) INFORMATION FOR SEQ ID NO: 7:


    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 21 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear


(ix) FEATURE:
        (A) NAME/KEY: modified_base
        (B) LOCATION:one-of(6, 10, 13-17)
        (D) OTHER INFORMATION:/mod_base= OTHER
                /note= "N = A, G, C, or T"


(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:


CGCUGNCCUN CCNNNNNGGG G                                          21



(2) INFORMATION FOR SEQ ID NO: 8:


    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 21 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
```

- 183 -

           (D) TOPOLOGY: linear

      (ix) FEATURE:
            (A) NAME/KEY: modified_base
            (B) LOCATION:one-of(6, 10)
            (D) OTHER INFORMATION:/mod_base= OTHER
                  /note= "N = A, G, C, or T"

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

    GCGACNGGAN GGAAAGACCC C   21

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CLAIMS

1.   A composition comprising an antimicrobial agent in
combination with a second agent that inhibits methylation
in a microorganism.

2.   The composition of claim 1, wherein said
antimicrobial agent is a macrolide lincosamide
streptogramin B (MLS) antibiotic.

3.   The composition of claim 2, wherein said MLS
antibiotic is a macrolide antibiotic.

4.   The composition of claim 3, wherein said MLS
antibiotic is erythromycin, azithromycin, clarithromycin,
roxithromycin, oleandomycin, spiramycin, josamycin,
miocamycin, midecamycin, rosaramycin, troleandomycin,
flurithromycin, rokitamycin or dirithromycin.

5.   The composition of claim 2, wherein said MLS
antibiotic is a lincosamide antibiotic.

6.   The composition of claim 5, wherein said MLS
antibiotic is lincomycin, clindamycin or celesticetin.

7.   The composition of claim 2, wherein said MLS
antibiotic is a streptogramin B antibiotic.

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8. The composition of claim 7, wherein said MLS antibiotic is pristinamycin or virginiamycin.

5   9. The composition of claim 1, wherein said second agent inhibits RNA methylation or maturation in a microorganism.

10   10. The composition of claim 9, wherein said second agent inhibits RNA methylation or maturation in a bacterium.

15   11. The composition of claim 10, wherein said second agent inhibits RNA methylation or maturation in an MLS-sensitive bacterium.

20   12. The composition of any one of claims 9-11, wherein said second agent inhibits RNA methylation.

13. The composition of claim 12, wherein said second
25   agent inhibits an RNA methyltransferase.

14. The composition of claim 13, wherein said second agent is S-adenosylhomocysteine (SAH), an SAH analogue,
30   homocysteine, an adenine derivative, a SAM analogue, a sulphonium SAM derivative, sinefungin, a sinefungin analogue, nicotinamide, a nicotinamide analogue or polyinosinate.

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15. The composition of claim 14, wherein said second agent is S-N^6-methyladenosylhomocysteine, S-aristeromycinyl-L-homocysteine, an S-aristeromycinyl-L-homocysteine analogue, SIBA,
5 S-Tubercidinylhomocysteine (STH), an STH analogue, A9145c, methylnicotinamide, MTA or xylosyladenine.

16. The composition of claim 12, wherein said second
10 agent inhibits S-adenosylhomocysteine (SAH) hydrolase (SAHH).

17. The composition of claim 16, wherein said second
15 agent is adenosine, an adenosine analogue, neplanocin A (NPA) or an NPA analogue.

18. The composition of claim 17, wherein said second
20 agent is (S)-DPHA), (D)-eritadenine, (RS)-AHPA, adenosine dialdehyde, C^{3}Ado, aristeromycin (Ari), an aristeromycin analogue, DHCeA, c^3-DHCeA, DHCaA, c^3-DHCaA, c^3-NpcA, c^3-Ari, 6'C-methylneplanocin A, 2'-deoxyadenosine, Ara-A or tubercidin.

25

19. The composition of claim 12, wherein said second agent inhibits S-adenosylmethionine (SAM) synthetase (SAMS).

30

20. The composition of claim 19, wherein said second agent is cycloleucine, a cycloleucine analogue, a methionine analogue or AMPNPP.

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21. The composition of claim 20, wherein said second
agent is L-*cis*-AMB, L-*cis*-AMTB or
5'(R)-(C)-[(L-homocysteine-S-yl) methyl]adenosine
5'-(β,γ)-imidotriphosphate.

22. The composition of claim 19, wherein said second
agent inhibits glutathione synthetase.

23. The composition of claim 22, wherein said second
agent is buthionine sulfoximine, 7,8-dihydrofolate,
α-aminomethylglutarate or SAPH-3.

24. The composition of claim 12, wherein said second
agent inhibits methionine synthetase (MS) or homocysteine
transmethylase.

25. The composition of claim 24, wherein said second
agent is nitrous oxide (N_2O).

26. The composition of claim 12, wherein said second
agent inhibits adenosine deaminase (ADA).

27. The composition of claim 26, wherein said second
agent is coformycin, a coformycin isomer, 1,6-dihydro-6-
hydroxy-methylpurine nucleoside,
erythro-9-(2-hydroxy-3-nonyl)adenine, 6-methylaminopurine
riboside or 2'-3'-isopropylidene-adenosine.

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28. The composition of claim 12, wherein said second agent inhibits dihydrofolate reductase (DHFR).

5   29. The composition of claim 28, wherein said second agent is methotrexate (MTX), aminopterin, trimethoprim, a 4,6-diamino-2,2-dimethyl-s-triazine analogue or a 2,4-diamino-5-(3,4-dichlorophenyl)pyrimidine analogue.

10

30. The composition of claim 28, wherein said second agent is a sulfonamide inhibitor of dihydropteroate synthetase.

15

31. The composition of claim 12, wherein said second agent inhibits polyamine synthesis.

20   32. The composition of claim 31, wherein said second agent is α-methylornithine, 1,3-diaminopropan-2-ol, DFMO or DFMA.

25   33. The composition of claim 31, wherein said second agent inhibits S-adenosylmethionine decarboxylase (SAM-DC).

30   34. The composition of claim 33, wherein said second agent is AMA, MHZPA, MAOEA, AbeAdo, AdoMac, MGBG, CGP-39'937 or CGP-33'829.

35   35. The composition of claim 31, wherein said second agent inhibits ornithine decarboxylase (ODC).

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36. The composition of claim 31, wherein said second agent inhibits arginine decarboxylase (ADC).

5

37. The composition of claim 9-11, wherein said second agent inhibits RNA maturation.

38. The composition of claim 37, wherein said second
10   agent is 5-azacitidine, cordycepin or toyocamycin.

39. The composition of any preceding claim, comprising between two and about five distinct antimicrobial agents
15   in combination with said second agent.

40. The composition of any preceding claim, comprising said antimicrobial agent in combination with between two
20   and about ten distinct second agents.

41. The composition of any preceding claim, comprising an MLS antibiotic in combination with a second agent and
25   a non-MLS antibiotic.

42. The composition of any preceding claim, wherein said composition is dispersed in a pharmaceutically acceptable
30   medium.

43. The composition of claim 42, wherein said pharmaceutically acceptable composition is suitable for
35   human administration.

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44. The composition of claim 42, wherein said
pharmaceutically acceptable medium is suitable for oral
administration.

5

45. The composition of claim 42, wherein said
pharmaceutically acceptable medium is suitable for
parenteral administration.

10

46. The composition of claim 42, wherein said
pharmaceutically acceptable medium is suitable for
topical administration.

15

47. The composition of claim 42, wherein said
pharmaceutically acceptable medium is suitable for nasal
administration.

20

48. The composition of claim 42, wherein said
pharmaceutically acceptable medium is suitable for
ophthalmic administration.

25

49. The composition of claim 42, wherein said
pharmaceutically acceptable medium is a liposomal
formulation.

30

50. An antimicrobial composition according to any
preceding claim, for use in reducing the resistance of a
microorganism to an antimicrobial agent.

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51. An antimicrobial composition according to any
preceding claim, for use in reducing the resistance of a
bacterium to an antibiotic.

5

52. An antimicrobial composition according to any
preceding claim, for use in reducing the resistance of an
MLS-susceptible bacterium to an MLS antibiotic.

10

53. An antimicrobial composition according to any
preceding claim, for use in inhibiting RNA methylation or
maturation in a microorganism.

15

54. An antimicrobial composition according to any
preceding claim, for use in inhibiting RNA methylation or
maturation in a bacterium.

20

55. An antimicrobial composition according to any
preceding claim, for use in inhibiting RNA methylation in
a microorganism or bacterium.

25

56. An antimicrobial composition according to any
preceding claim, for use in inhibiting RNA maturation in
a microorganism or bacterium.

30

57. An antimicrobial composition according to any
preceding claim, for use in killing a microorganism.

35   58. An antimicrobial composition according to any
preceding claim, for use in killing a bacterium.

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59. An antimicrobial composition according to any
preceding claim, for use in killing an MLS-susceptible
bacterium.

5

60. An antimicrobial composition according to any
preceding claim, for use in reducing the resistance of a
microorganism within an animal to an antimicrobial agent
10   wherein the composition is administered to the animal in
an amount effective to reduce the resistance of the
microorganism to the antimicrobial agent.

15   61. An antimicrobial composition according to any
preceding claim, for use in killing a microorganism
within an animal wherein the composition is administered
to the animal in an amount effective to kill the
microorganism.

20

62. An antimicrobial composition according to any
preceding claim, for use in treating a microbial
infection wherein the composition is administered to an
25   animal with a microbial infection in an amount effective
to reduce said infection.

63. An antimicrobial composition according to any
30   preceding claim, for use in treating a bacterial
infection wherein the composition is administered to an
animal with a bacterial infection in an amount effective
to reduce said infection.

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64. An antimicrobial composition according to any
preceding claim, for use in treating a human subject with
a microbial or bacterial infection wherein the
composition is administered to the human subject in an
5 amount effective to reduce said infection.

65. Use of a composition according to any preceding
claim in the manufacture of a medicament for treating a
10 microbial infection in an animal wherein the medicament
is administered to said animal in an amount effective to
reduce said microbial infection.

15 66. Use of an agent that inhibits methylation in a
microorganism in the manufacture of a medicament for
treating a microbial infection in an animal wherein the
medicament is administered to said animal in an amount
effective to inhibit RNA methylation or maturation in a
20 microorganism.

67. A use according to claim 66, wherein said agent is
formulated into a medicament for administration to an
25 animal prior to administration of an anti-microbial
agent.

68. A use according to claim 66, wherein said agent is
30 formulated into a medicament for administration to an
animal subsequent to administration of an anti-microbial
agent.

35 69. A use according to claim 66, wherein said agent is
formulated into a medicament for administration to an

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animal simultaneously with administration of an anti-
microbial agent.

5   70.   A use according to claim 66, wherein said agent is
formulated into a single medicament in combination with
an anti-microbial agent.

10   71.   A kit comprising, in suitable container means, a
pharmaceutical formulation of at least one antimicrobial
agent and a pharmaceutical formulation of at least one
microbial methylation inhibitor.

15

72.   The kit of claim 71, wherein said antimicrobial
agent and said methylation inhibitor are present within a
single container means.

20

73.   The kit of claim 71, wherein said antimicrobial
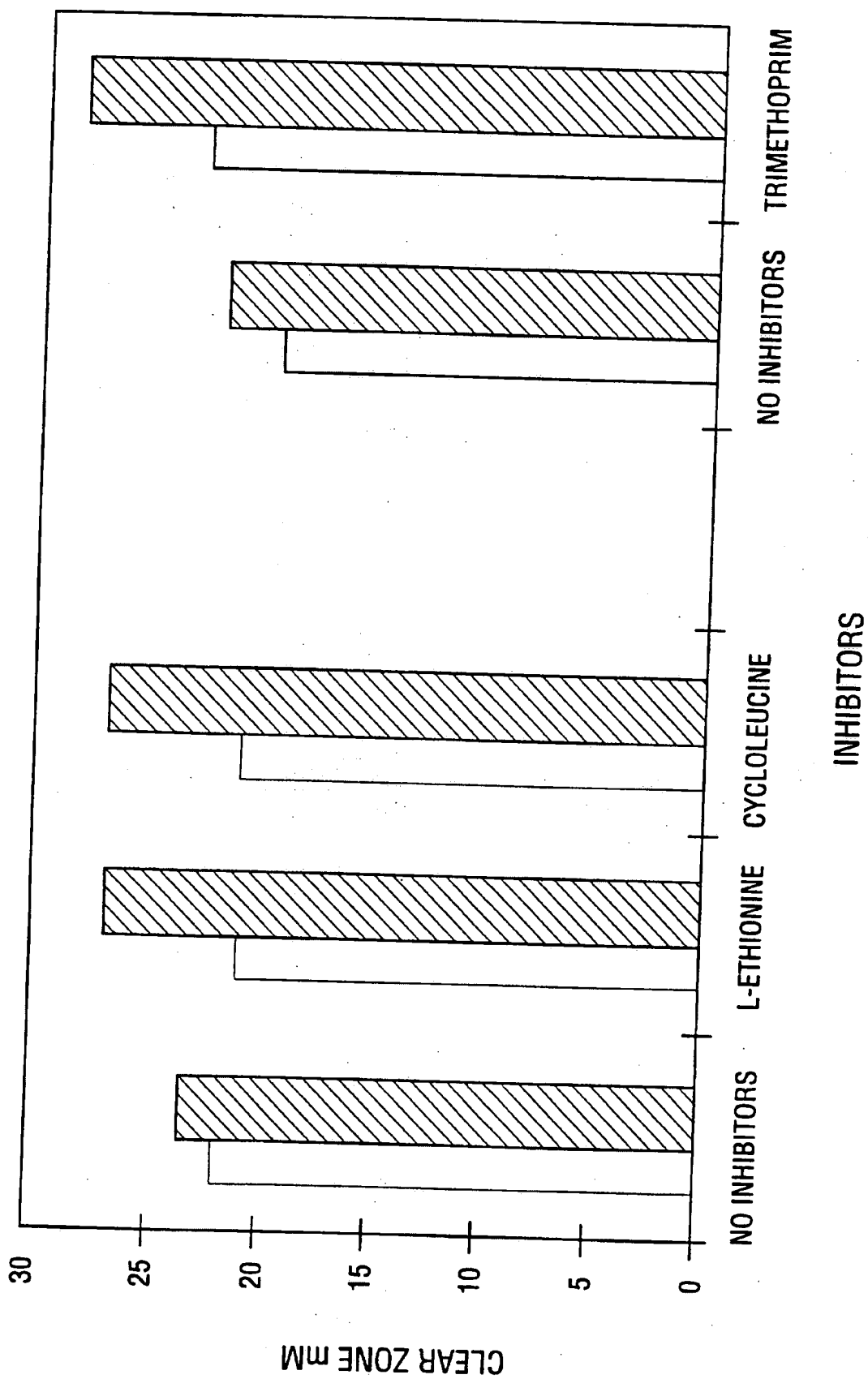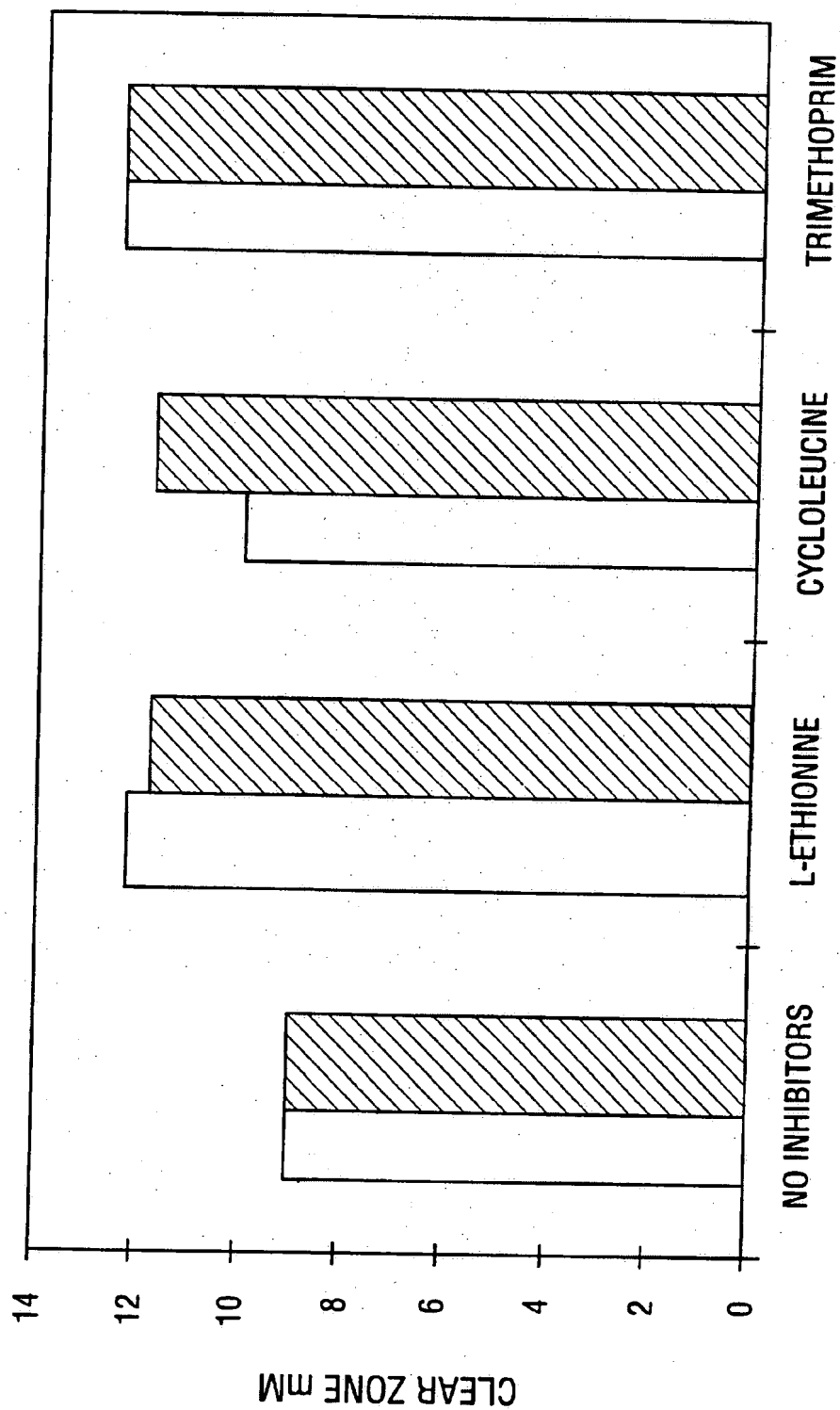agent and said methylation inhibitor are present within
distinct container means.

FIG. 1

FIG. 2

FIG. 3

INTERNATIONAL SEARCH REPORT
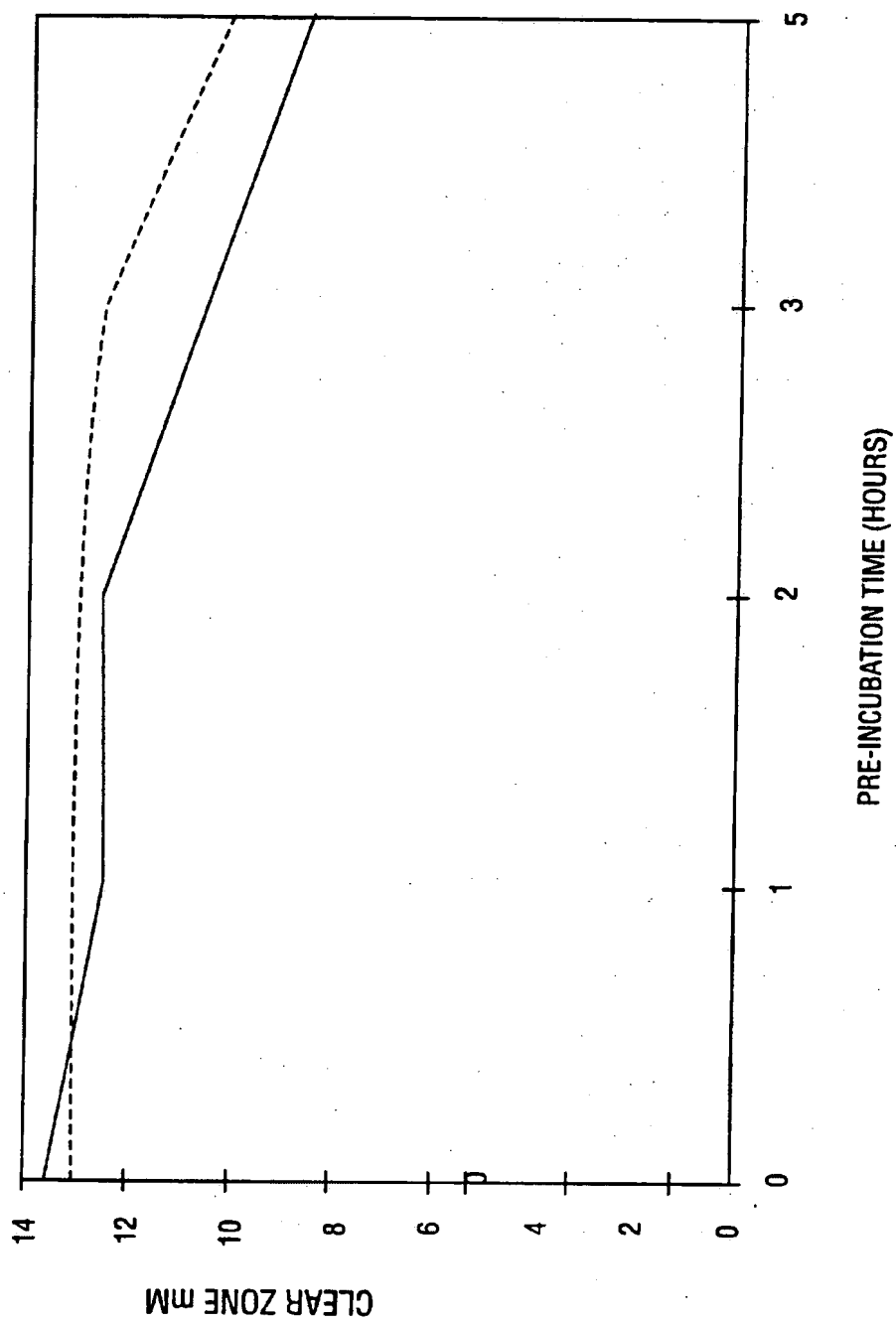
A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   A61K45/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 052 705 (BERNSTEIN JOEL E) 2 June 1982 see claims 1,2 ---	1-6,9-14
X	J ANTIBIOT (TOKYO), 39 (9). 1986. 1291-1297., XP000571520 HASOBE M ET AL: "THE SYNERGISM OF NUCLEOSIDE ANTIBIOTICS COMBINED WITH GUANINE-7-N-OXIDE AGAINST A RHABDOVIRUS INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS" see page 1296, paragraph 4-7 ---	1,9-18
X	PRESSE MED., 1994, 23/26 (1195-1198), FRANCE, XP000571949 TOUZE J.E.: "New therapeutic approaches to anti-parasite treatments" see abstract ---	1,9-12, 28

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

29 May 1996

Date of mailing of the international search report

06.06.96

Name and mailing address of the ISA
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Fax (+31-70) 340-3016

Authorized officer

Leherte, C

Form PCT/ISA/210 (second sheet) (July 1992)

Internatic   Application No

PCT/US 95/16677

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. INFECT. DIS., 1987, 155/5 (1027-2032), USA, XP000571952 KOVACS J.A. ET AL: "Potent effect of trimetrexate, a lipid-soluble antifolate, on Toxoplasma gondii" see abstract -----	1,9-12, 28

1

INTERNATIONAL SEARCH REPORT

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:   1-73
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

   Please see annex!

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee. this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

                                ☐ No protest accompanied the payment of additional search fees.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0052705	02-06-82	US-A- 4505896	19-03-85
		AU-B- 7554481	03-06-82
		CA-A- 1176986	30-10-84